



# **EFFECTS OF HIGH FAT DIET ON COGNITION AND BRAIN PATHOLOGY**

A thesis submitted in fulfilment of the requirements for the Master by Research

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## **Declaration**

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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## Abbreviations

Acetyl CoA	Acetylcoenzyme A
AchE	Acetylcholinesterase
A $\beta$	Amyloid-beta
BMI	Body mass index
CA1	Cornu Ammonis 1
CA2	Cornu Ammonis 2
CA3	Cornu Ammonis 3
ChAT	Choline acetyltransferase
Con	Control
DAB	3,3-diaminobenzadine hydrochloride
DG	Dentate gyrus
DR	Discrimination ratio
GFAP	Glial fibrillary acidic protein
GTT	Glucose tolerance test
HF	High fat
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IEG	Immediate-early gene
IL-1 $\beta$	Interleukin 1 $\beta$
IL-6	Interleukin 6
MS	Medial Septum
NHMRC	National Health and Medical Research Council
NGS	Normal goat serum
PBS	Phosphate buffered saline

Rdg	Retrosplenial dysgranular cortex
RTK	Receptor tyrosine kinase
Rga	Retrosplenial granular a cortex
Rgb	Retrosplenial granular b cortex
VDBD	Vertical limb of the diagonal band
WD	Western diet
WHO	World Health Organization

## Summary

Consumption of a high fat diet in western countries has increased significantly over the past few decades, leading to major health issues. Recent evidence shows an association between obesity and cognitive decline. The present studies aimed to (i) determine whether high fat or western diet can affect working or spatial memory in rats (ii) investigate the changes that occur in brain areas important for memory regulation following the consumption of a higher percentage of fat in diet.

Male Long Evan and Hooded-Wistar rats were fed normal chow, western diet (23% fat, 0.19% cholesterol) or high fat diet (60% fat) for 12 weeks. Body weight and food intake were measured twice weekly. Behavioural testing was carried out after 12 weeks of feeding by investigating (i) working memory performance in the novel object recognition test, (ii) spatial memory using a Y-maze and (iii) rewarded alternation using a T-maze. In one cohort of rats the Y-maze and novel object recognition tasks were repeated at the end of each month of feeding for three months to find out at what point during the feeding, the memory impairment begins. Immunohistochemistry was carried out in specific brain areas including those that are important in memory regulation in order to investigate the changes in the level of cholinergic markers, basal neuronal activity, astrocytes and insulin like growth factor 1 receptor.

This study is the first to report memory impairment using a Y-maze paradigm (which is a one trial-one test procedure) as the result of higher percentage of fat in diet in rats (Kosari et al., 2012). The impaired spatial memory observed in the high fat group was independent of changes in body weight or blood pressure. Results revealed no significant difference in the working memory performance of rats that consumed



control, western or high fat diets for three months using novel object recognition task. No significant difference was observed in the T-maze performance between the groups that consumed a western diet (21% fat) or a control diet (6% fat).

The immunohistochemistry results revealed a significant increase in the level of acetylcholinesterase (an enzyme responsible for the breakdown of acetylcholine) in the striatum of the rats that consumed a high fat diet (60% fat) compared to Long Evan rats fed a control diet. Further analysis revealed no difference in the number of choline acetyltransferase (ChAT)-positive cells (ChAT is an enzyme responsible for production of acetylcholine) in the medial septum and vertical limb of the diagonal band (both are areas in forebrain that are believed to be important in cognitive function) of the rats that consumed any of the three diets.

The consumption of a western diet for twelve weeks significantly decreased basal c-fos immunoreactivity in the CA2&3 regions of the hippocampus, prelimbic and infralimbic regions of the prefrontal cortex in other cohorts of Long Evan rats. This indicates decreased neuronal activity in those areas of the brain. Moreover, basal c-fos immunoreactivity decreased significantly following consumption of a western diet in the paraventricular nucleus of thalamus which has extensive connections with the prefrontal cortex and hippocampus.

Some studies reported changes in c-fos expression consistent with the changes in the level of insulin like growth factor 1 (IGF-1). Therefore, I investigated the changes in the level of IGF-1 receptor in the hippocampus, prefrontal cortex and retrosplenial cortex in the group of Hooded-Wistar rats that consumed western or control diet for three months. The results revealed no significant differences between the two groups.

This is the first study that investigated the potential changes that may occur in astrocytes in the regions of the brain important for cognition and memory following western diet consumption in adult rats. Immunohistochemistry results revealed that the number of glial fibrillary acidic protein (GFAP) positive cells (as an astrocyte marker) was decreased significantly in CA1 and dentate gyrus of the hippocampus of rats that consumed a western diet for three months. These results suggest a decrease in the number of GFAP-positive astrocytes as the result of consumption of a western diet. However, no change was observed in the GFAP immunohistochemistry staining after three months of western diet consumption in the areas of the prefrontal cortex.

In conclusion, this research demonstrated an impairment of spatial memory in rats following the consumption of a western or high fat diet for three months. Further investigation revealed that consumption of these diets is associated with changes in specific areas of the brain including those that are involved in memory regulation. These changes include the increased level of acetylcholinesterase in the striatum, the reduced number of GFAP-positive astrocytes in CA1 and dentate gyrus of the hippocampus besides the decreased level of basal neuronal activity in CA2&3 region of the hippocampus, prelimbic and infralimbic areas of the prefrontal cortex and in the paraventricular nucleus of thalamus in rats following consumption of high fat or western diets. Overall, these results suggest that higher percentage of fat in diet can affect specific regions of the brain that are important for memory regulation through various mechanisms and consequently may affect the memory. Further studies are necessary to elucidate the precise mechanisms that are involved in the association between the consumption of a western diet and changes in the brain that consequently may affect memory function.

# **1. Chapter 1: Introduction**

## **1.1. Obesity and its prevalence**

A significant increase in consumption of high fat and high sugar food contributes to greater body weight and subsequently to the obesity epidemic around the world. Obesity is defined as a chronic metabolic disorder, a consequence of excessive fat accumulation in the body and an imbalance between energy intake and energy expenditure. Assessment of obesity was started by measuring height and weight. In 1997, the World Health Organization (WHO) defined obesity as a body mass index (BMI);  $\text{weight (kg)/height (m)}^2 \geq 30 \text{ kg/m}^2$  (World Health Organization, 1995, 2000) and overweight is defined as the  $\text{BMI} \geq 25 \text{ kg/m}^2$  (World Health Organization, 2015).

In 2014, more than 1.9 billion adults (18 years and older) were overweight and among them 600 million were obese. This number puts 39% of adults in the overweight category (38% of men and 40% of women) and 13% in the obese category (11% of men and 15% of women) (World Health Organization, 2015). It has been predicted that about 700 million people will be obese in 2015 worldwide (World Health Organization, 2011). According to the World Health Organization, obesity prevalence has doubled since 1980 worldwide and more than 42 million children under five years old have been reported as overweight or obese in 2013 (World Health Organization, 2015). Currently the fifth leading risk for global death is considered overweight and obesity (World Health Organization, 2015). An Australian Health Survey reported a rise in the prevalence of overweight and obese adults aged 18 years and over in Australia from 56.3% in 1995 to 63.4% in 2011-12 (Australian Bureau of Statistics, 2012). Moreover, this rise in the number of obese individuals contributes significantly

to increase of healthcare expenditure for obesity and its related health issues (Lehnert et al., 2013).

## **1.2. Complications associated with obesity and high fat diet in humans**

In conjunction with reducing life expectancy (Stevens et al., 1998, Calle et al., 1999, Peeters et al., 2003), overweight and obesity can contribute to high levels of cholesterol and triglyceride and high blood pressure (Denke et al., 1993, Brown et al., 2000, Wilson et al., 2002). Moreover, studies have reported that obesity and consumption of a high fat diet (even in the absence of obesity) can cause insulin resistance (Swinburn, 1993, Marshall et al., 1994, Oakes et al., 1997, Kahn and Flier, 2000) and lead to an increase in the risk of type 2 diabetes (Mokdad et al., 2003, Gregg et al., 2004), cardiovascular disease, atherosclerosis, stroke (Wilson et al., 2002, Sowers, 2003, Lavie et al., 2014), cancer (Calle et al., 1999, Adami and Trichopoulos, 2003, Calle et al., 2003) and osteoarthritis (Anderson and Felson, 1988, Felson et al., 1988, Blagojevic et al., 2010).

### **1.2.1. Effect of obesity on cognition in humans**

#### **1.2.1.1. Cognitive decline, dementia and its prevalence**

Cognition is a broad term that includes a wide range of mental processes such as understanding, memory, thinking, reasoning, attention, visuospatial ability and language (Morris et al., 1999a) (Cognition and memory are further explained in section 1.4). A patient will be diagnosed with dementia when there is long lasting impairment in most of those abilities (American Psychiatric Association, 1994). Recently there has

been an increase in the prevalence of dementia in both developing and developed countries affecting the economy, creating a global concern and a major public health issue (Berr et al., 2005, Plassman et al., 2007, Prince et al., 2012). The World Health Organization reported that in 2012, 36.6 million people worldwide lived with dementia. This number was 44 million in 2013 and 7.7 million more will suffer from this disease each year (World Health Organization, 2012, Alzheimer's Disease International, 2013). It has been predicted that every 20 years, this growth in the number of people diagnosed with dementia will be doubled and by 2050 will reach to 135 million patients (Alzheimer's Disease International, 2013).

There are different types of dementia including Alzheimer's disease, vascular dementia and dementia with Lewy body. About 70% of cases with dementia are diagnosed with Alzheimer's disease (Alzheimer's Association, 2013). In Alzheimer's disease the cognitive ability will gradually decline in a progressive manner and has two main characteristics: One is the presence of amyloid-beta ( $A\beta$ ) plaques that are produced due to the over production of abnormal  $A\beta$  protein and the second one is the existence of neurofibrillary tangles that are abnormal phosphorylated tau proteins (Hauw et al., 1990, Pitt et al., 2013). Currently there is no effective cure for dementia. Public health institutions consider dementia as a global health priority (World Health Organization, 2012). Therefore there is a great need to find and implement strategies to prevent dementia. Obesity and high fat diet consumption is one of the potential factors that may influence the prevalence of this pressing public health concern.

#### **1.2.1.2. Obesity and dementia in human**

Numerous studies in humans have shown that metabolic and cardiovascular risk factors such as obesity, hypertension and diabetes are associated with accelerated cognitive ageing and increased risk of developing dementia.

In 1982, Sorensen et al. for the first time reported that severe obesity is strongly associated with reduced intellectual performance in a population of Danish young men (Sorensen et al., 1982). Kilander et al. in 1997 reported that obesity coupled with other factors including hypertension, diabetes, cholesterol or smoking is associated with cognitive decline (Kilander et al., 1997). In 2003, Elias and colleagues demonstrated an adverse effect of obesity and hypertension together, on multiple cognitive measures (Elias et al., 2003). Further analysis revealed that this decrease in cognitive function observed in obesity was independent of associated variables such as hypertension, smoking and other factors (Elias et al., 2005). Consistent with these findings, studies of young obese adults that were otherwise healthy confirmed the inverse association of body mass index with cognitive functions such as memory and executive functioning (Cournot et al., 2006, Gunstad et al., 2007). Abdominal obesity also has been reported by Gunstad et al. to be directly associated with cognitive impairment (Gunstad et al., 2010).

Several longitudinal population-based studies provided further evidence of the effect of body weight on cognitive decline. In 2005, Kivipelto et al. in a population-based study showed that obesity in midlife is associated with an increased risk of dementia and Alzheimer's disease later in life (Kivipelto et al., 2005). In addition the Finish Twin Cohort Study conducted on 2606 twin individuals demonstrated that the higher BMI in midlife even a subtle weight change may lead to cognitive impairment during aging

(Laitala et al., 2011). Furthermore, participants both men and women in the Swedish Adoption/Twin Study Aging showed an association of higher BMI in midlife and lower cognitive ability over a time period of about 40 years (Dahl et al., 2010). This is consistent with other research such as a Swedish research showing women with higher BMI at ages 70 to 79 years develop subsequent dementia (Gustafson et al., 2003) or another aging study demonstrating an increased risk for vascular dementia with increasing BMI in the male cohort of the Honolulu-Asia (Kalmijn et al., 2000). The similar results have been reported in a multiethnic population-based study that revealed overweight and obese men and women had 35% and 74% increased risk of developing dementia respectively compared with those that had normal body weight (Whitmer et al., 2005). Interestingly, in a comparison study, Hayden and colleagues showed that in a cohort of 3264 community members aged 65 and older, obesity increased the risk of Alzheimer's disease in females but not males (Hayden et al., 2006).

More recent observational studies have moreover revealed an association between BMI and cognition, suggesting that age of the population plays a role in this correlation. For example in younger adults, higher BMI is associated with impaired cognition. In middle age, obesity is linked to increased risk of future dementia in older age (Whitmer et al., 2005, Dahl et al., 2013, Dahl Aslan et al., 2014, Faxen-Irving et al., 2014, Nepal et al., 2014).

### **1.3. Rodent models of diet-induced obesity**

In order to study obesity and its complications, different animal models have been developed including genetic models and diet-induced obesity models.

### **1.3.1. Genetic models**

Genetic rodent models have been developed to study obesity including those that are the result of spontaneous single gene mutation or knockout models (Farooqi and O'Rahilly, 2005, Rankinen et al., 2006). Leptin-deficient ob/ob mice are the result of a single-base spontaneous mutation of the ob gene causing the inhibition of leptin secretion by preventing its synthesis (Zhang et al., 1994). Leptin is an important protein secreted by adipocytes that acts on the hypothalamic leptin receptors and reduces food intake and increases energy expenditure (Zhang et al., 1994). db/db mice are leptin receptor-deficient (Bahary et al., 1990) and Zucker (fa/fa) obese rats carry mutated forms of the extracellular domain of the leptin receptor (Zucker and Zucker, 1961). In addition, many transgenic and knockout rodent obese models have been developed in which specific gene is disrupted or over-expressed (Inui, 2000, Salton et al., 2000, Rankinen et al., 2006).

### **1.3.2. Cafeteria diet fed model**

The term Cafeteria diet is used for a variety of palatable high energy and high sugar food similar to western food (Sclafani and Springer, 1976). Cafeteria diet has been used for varying durations in adolescent, adult and aged animals (Rogers and Blundell, 1984, Perez et al., 1999, Hansen et al., 2004, Morris et al., 2008, Heyne et al., 2009, Shafat et al., 2009, Caimari et al., 2010). Consumption of a cafeteria diet for 8 weeks caused an increase in body weight and leptin level in rats and no change in glucose, insulin or triglyceride level (Lopez et al., 2003). Male Hooded-Wistar rats that consumed a cafeteria diet including high salt, high fat, low fibre and high energy dense foods for 15 weeks exhibited a higher body weight compared to high fat fed (45% fat in diet) and control rats (Sampey et al., 2011). Moreover, those rats showed



exaggerated hyperinsulinemia, hyperglycaemia and glucose intolerance (Sampey et al., 2011). Several other studies reported similar results as well as changes in brain regions important for memory (Hansen et al., 2004, Martire et al., 2014).

### **1.3.3. High fat fed model**

The term “high fat” diet is used for a variety of diets with a higher percentage of fat (20% - 85% energy from fat) compared to a normal diet (less than 20% and usually 6% energy from fat) (Buettner et al., 2007, Hariri and Thibault, 2010). This diet includes various basic fat components that are derived from animals or plant oils (Buettner et al., 2007). The effects of the wide range of diets in rodents are diverse. While some rodents do not become obese even if they consume a high fat diet (Sclafani and Springer, 1976, Levin et al., 1983, Chang et al., 1990), in most of the studies using both rats and mice, higher percentage of fat in diet resulted in a higher body weight (Rothwell and Stock, 1984, Warwick and Schiffman, 1992, Boozer et al., 1995, Takahashi et al., 1999, Buettner et al., 2007). The observed increase in body weight can be due to higher energy intake (Ghibaudi et al., 2002) and/or weaker satiety signals from fat compared with carbohydrates and proteins (Blundell and MacDiarmid, 1997) that leads to more energy intake. This can change the level of hormones important in energy balance including an increase in leptin (Leibowitz et al., 2006) and insulin levels (Woods et al., 2004) and in some cases can cause insulin and leptin resistance (Riccardi et al., 2004, Woods et al., 2004).

Studies that used several weeks of a high fat diet in both rats and mice have generally observed moderate hyperglycemia and an increase in fasting triglyceride levels (Woods et al., 2004, Buettner et al., 2007, Hariri and Thibault, 2010). Further research revealed

that the type of fat used in the high fat diet is important in developing obesity. Studies revealed that consumption of saturated fatty acids is more obesogenic compared to polyunsaturated fatty acids (Storlien et al., 1998, Wang et al., 2002, Hariri et al., 2010).

## **1.4. Cognition/Memory**

Cognitive function includes the acquisition, processing, integration, storage and retrieval of information. Cognitive theory argues that memory is supported by multiple cognitive and neuronal systems including spatial and working memory (Baars and Gage, 2010). Learning and memory have been defined respectively as the abilities to acquire new information and to retain it over time.

### **1.4.1. Spatial memory**

Spatial memory cannot be strictly assigned to only one category, and is responsible for recognizing, coding, storing and recovering spatial information about the configurations of objects or routes as the result of exploration (Kessels et al., 2001, Paul et al., 2009). This type of behaviour can be considered an expression of natural curiosity or may represent the need to acquire information when subjects face a new environment and new stimuli. One of the paradigms used to study spatial memory in rodent is the Y-maze that is based on the spontaneous inclination of a rat to explore novel places and objects (Dember and Fowler, 1958, Poucet et al., 1986, Crusio et al., 1989) without using any motivator (further explained in chapter 2).

### **1.4.2. Working memory**

Working memory includes the mental processes that are involved when information is retained over a period of seconds to minutes. It represents a limited-capacity store for retaining information over the short term (maintenance) and for performing mental operations on the contents of this store (manipulation). Remembering a series of words and recalling them at a later time is an example of working memory in humans (Gazzaniga et al., 2009).

## **1.5. Memory paradigms**

Different memory paradigms have been developed in order to investigate various memory types. A few examples of the common memory tasks are discussed below.

### **1.5.1. Alternation paradigms**

One measure of exploratory and memory behaviour is spontaneous alternation in animals including rodents (Dember and Fowler, 1958, 1959) that is based on the tendency of animals not to repeat their recent response (Tolman, 1925, Gerlai, 1998). In alternation tasks usually there are two phases, in the first phase the animals are allowed to enter only one of the arms of the Plus, Y or T-maze. On the second phase the animals can enter the same arm or choose to alternate and enter a new arm. The first phase can be a forced-trial in which one of the arms is blocked or free choice trial in which the rodent is free to explore any arm. Studies showed that rodents tend to alternate more in a forced-trial paradigm as the animals prefer to enter the unfamiliar arm if in the past they were forced to enter only one arm (Dember and Fowler, 1958, 1959).

The T-maze is the most common version of the alternation paradigm. Reinforced alternation is a short-term memory task in which the animal will be exposed to the apparatus and needs to choose alternative maze arms or locations in general for a food reward (reinforcement) (Richman et al., 1986). If the alternation is acquired by repeating the tasks and rewarding or punishing the rodent for correct or incorrect entries successively, learned alternation takes place (Means et al., 1971, Thomas, 1972, Thomas and Spafford, 1984).

The alternation tendency of rats applies to the radial arm maze as well in which rats placed in the centre of commonly an eight-arm maze and required to obtain food from the end of each arm without re-entering an arm that it has already entered. The correct entry to the arm shows that rodent have a good spatial memory for the arms that have been visited or not yet entered. Therefore it is used as a test for the spatial memory. In order to test the working memory by the radial maze, entry to only four arms are allowed and the other four are blocked. After a delay, all eight arms are made open and animals are expected to enter only those arms that were not visited before (Buresova et al., 1985, Roberts, 2006, Paul et al., 2009).

In order for an animal to choose this alternative response, they need to remember their initial response and their exterior environment. In this task animal must integrate multiple cues in a novel environment to learn a new task. This is a hippocampal-dependent learning task (Dudchenko, 2004) as lesions of the hippocampus caused a decrease in spontaneous alternation in the T-maze free-trial task (Means et al., 1971, Stevens and Cowey, 1973, Johnson et al., 1977). Lesions to other parts of the brain including septum (Douglas and Raphelson, 1966, Clody and Carlton, 1969), prefrontal

cortex (Divac et al., 1975) and dorsal striatum (Divac et al., 1975) resulted in a decrease in alternation rate.

### **1.5.2. Morris water maze**

The Morris water maze is a task of spatial memory in which the rodent must learn the location of a hidden platform in a round pool filled with opaque water using spatial cues throughout the room in order to escape from the pool (Morris et al., 1982). Rodents learn the location of the platform after a number of trials from four starting points and normally each trial takes a shorter time to find the platform (Sharma et al., 2010). In contrast, working memory can be tested in the Morris maze by moving the platform position each day so the animal has to re-learn the “rule” of platform position (Morris, 1984).

## **1.6. Memory and Brain Structures**

Different brain structures support various memory processes depending on the type of information to be retained and how it is encoded and retrieved (Baars and Gage, 2010).

### **1.6.1. Hippocampus**

The medial temporal lobe including the hippocampal region and the adjacent perirhinal, entorhinal, and parahippocampal cortices are essential in cognition and memory (Squire et al., 2004). Studies showed that, the hippocampus including the CA1 (cornu ammonis 1), CA2&3 (cornu ammonis 2&3) and dentate gyrus (DG) are specifically important for spatial location information and lesion of the hippocampus

causes impairment in spatial navigation tasks in rodents (Morris et al., 1982, Long and Kesner, 1996, Whishaw et al., 1997, Long and Kesner, 1998, Moser and Moser, 1998).

### **1.6.2. Retrosplenial cortex**

It is more than a century that the human retrosplenial cortex was defined as an area in the posterior cingulate by Brodmann (Brodmann, 1909). It is located behind the splenium around the corpus callosum. In rats, the retrosplenial cortex is one of the largest cortical regions, and covers about half of the length of the entire cerebrum. According to Van Groen and Wyss, this area can be divided into dysgranular (Rdg), granular a (Rga) and granular b (Rgb) regions (van Groen and Wyss, 1990, 1992, 2003). However, not much was known about its function and it was believed to be mainly involved in regulating emotion (Papez, 1995, Vogt et al., 2000). Recently studying the connections of retrosplenial cortex to other brain regions reveals its role in memory; there are reciprocal connection between the retrosplenial cortex and those regions important for memory such as hippocampal formation, parahippocampal regions and anterior and lateral dorsal nuclei of thalamus. There are denser hippocampal and anterior thalamic projections to retrosplenial cortex compared to other areas of the posterior cingulate, suggesting a role for the retrosplenial cortex in hippocampus-dependent functions. Moreover, there are reciprocal pathways between the retrosplenial cortex and parts of prefrontal cortex that serve as an indirect path between the hippocampus and dorsolateral prefrontal cortex: an important cortical area involved in executive function (Morris et al., 1999b).

The granular a and granular b subregions of the retrosplenial cortex have connections with the areas containing head-direction cells, however, the dysgranular area of

retrosplenial cortex is more connected with visual areas (van Groen and Wyss, 1992, Jones et al., 2005). Rats with lesions of retrosplenial granular b cortex (Rbg) were slightly, but significantly, impaired in a repeated acquisition water maze task (van Groen et al., 2004). Interestingly destruction of retrosplenial granular a and b cortex at the same time reported to cause a much greater impairment in learning than would be predicted from their independent contributions (Vann et al., 2009). Selective lesions in Rdg subregion caused impairment in the use of visual cues in the radial arm maze in rodents (Vann and Aggleton, 2005). Function of retrosplenial cortex is linked with spatial navigation, episodic memory and planning the future and its dysfunction can effect different pathological conditions including Alzheimer's disease and cognitive dysfunction (Vann et al., 2009). Recent study in rodents revealed the role of retrosplenial cortex in encoding and storing spatial information and long term spatial memory due to its interaction with the hippocampus (Czajkowski et al., 2014, Miller et al., 2014).

### **1.6.3. Prefrontal cortex**

The prefrontal cortex consists of interconnected neocortical areas that are connected with almost all the sensory neocortical and motor and subcortical structures and has projections back to those systems. According to Zilles's terminology (Zilles, 1985), prefrontal cortex can be divided to two areas of medial and lateral regions and the medial prefrontal cortex is further divided to the three areas of the anterior cingulate, the prelimbic and the infralimbic. In recent studies in rat, prefrontal cortex has been distinguished by four different subregions of (i) medial, (ii) ventral medial, (iii) lateral and (iv) ventral prefrontal cortex (Uylings and van Eden, 1990, Groenewegen and Uylings, 2000). Each of those subregions is interconnected with different areas of the

brain. (i) The medial prefrontal cortex, consists of a dorsal medial region including the precentral cortex, the dorsal and ventral anterior cingulate cortices is connected with neocortex areas such as medial dorsal thalamic nuclei, intralaminar thalamic nuclei and nucleus accumbens (Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007). (ii) The ventral medial prefrontal cortex, is subdivided into the prelimbic, infralimbic, and medial orbital cortices and is connected with the limbic areas such as the perirhinal cortex, entorhinal cortex, hippocampus, basal nucleus of the amygdala and medial basal forebrain and to areas such as the medial dorsal nucleus, parataenial nucleus of the thalamus, midline thalamic nuclei and nucleus accumbens (Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007). (iii) The lateral prefrontal cortex is divided to the dorsal and ventral agranular insular and the lateral orbital cortices that are connected with different areas including olfactory bulb, parietal cortex, perirhinal cortex, the medial dorsal nucleus and central medial nucleus of the thalamus (Reep et al., 1996, Ongur and Price, 2000). (iv) The ventral prefrontal cortex is divided into the ventral orbital and ventrolateral orbital cortices and those are associated with areas such as parietal cortex, visual association cortex, medial dorsal nucleus, and central medial nucleus of the thalamus and posterior parietal cortex (Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007). These different areas of the prefrontal cortex, each project to different parts of the brain (Reep et al., 1996, Ongur and Price, 2000, Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007).

These patterns of connectivity enable this region to integrate a wide range of information required for complicated behaviour and influence many brain processes. Prefrontal cortex is particularly well known for its involvement in executive function such as temporal processing, planning, flexibility, decision making and specifically in working memory (Miller, 2000, Miller and Cohen, 2001) and its connection with the



hippocampus (Mendez et al., 2008, Kesner and Churchwell, 2011). Studies showed that although lesions of the prelimbic–infralimbic/medial orbital cortex could not cause a working memory deficit for a food reward (DeCoteau et al., 1997, Ragozzino et al., 1999), however, it could cause working memory impairment for spatial information (Brito and Brito, 1990, Granon et al., 1994, Seamans et al., 1995, Delatour and Gisquet-Verrier, 1996, Fritts et al., 1998, Ragozzino et al., 1998, Horst and Laubach, 2009) and deficit in working memory for visual object information (Kesner et al., 1996, Ragozzino et al., 2002, Di Pietro et al., 2004).

#### **1.6.4. Other brain regions involved in cognition**

Anterior thalamic nuclei and nucleus accumbens are important in memory due to the pathways existing between the hippocampal formation, prefrontal cortex, amygdala and those brain regions (Yasoshima et al., 2007, Aggleton et al., 2010).

The medial septum (MS) and the vertical limb of the diagonal band (VDBD) in forebrain are believed to be important in cognitive function specifically memory regulation mainly due to their projections to the hippocampus (Gaykema et al., 1991, Gallagher and Colombo, 1995).

### **1.7. High fat diet and its effect on memory tasks**

#### **1.7.1. Studies in Mice**

Different mice strains, ages and diets have been used to study the effect of a high fat diet in mice. For example C57BL/6J mice that were fed a high fat diet (45 kcal% from fat) for 8 weeks spent more time learning a radial arm maze task and made a greater

number of errors than controls diet (10 kcal% from fat). The same diet-induced obese mice made more working and total memory errors as evaluated by retention tests demonstrating spatial learning impairment in diet-induced obesity (Valladolid-Acebes et al., 2011). In another study, aged (20-month old) C57Bl/6 mice were fed a western diet (41% fat), very high fat lard diet (60% fat), or corresponding control diets for 16 weeks. Only a very high fat lard diet impaired retention in the behavioural test as measured by the T-maze (Morrison et al., 2010), however, the consumption of a western diet for 21 weeks by C57Bl/6 mice increased their body weight but did not impair their performance in the T-maze. In the same study consumption of the very high fat lard diet for 16 weeks increased body weight, and impaired cognition as tested by Stone T-maze. In this paradigm rodents need to learn the correct sequence of 13 consecutive left and right turns to reach the goal box and successfully escape the maze. The maze is maintained in a tray of water filled to a level that allows the animals to keep their head out of the water while maintaining contact with the floor, but the height of the top prevents rearing therefore the rodents are motivated to wade in order to escape the maze. In this paradigm several brain structures are involved including the hippocampus and parietal cortex (Pistell et al., 2009, Pistell et al., 2010).

### **1.7.2. Studies in Rats**

#### **1.7.2.1. Spatial memory**

Consumption of diets with increased sugar and fats has been conducive to impairment in spatial memory (Stranahan et al., 2008, Ross et al., 2009, Valladolid-Acebes et al., 2011). Female Fischer rats, one and two months after being fed a high fat, refined sugar diet showed spatial memory deficits in the water maze (Molteni et al., 2002) while a similar diet for five months, produced spatial reference memory deficits in radial arm

maze in adult male rats (Ullrich et al., 2010). This impairment in spatial memory after a high fat and sugar diet has been reported in young (Goldbart et al., 2006) and aged (Granholt et al., 2008) rats as well. A 20% lard-based diet (Greenwood and Winocur, 1990, 1996) and 58% high fat diet (Pathan et al., 2008) consumed by young rats, produced spatial memory deficits as tested in Morris water maze.

To examine the effect of different high-fat dietary treatments on memory in rats, one study placed separate groups of young (1-month old) Long-Evans rats on three different diets. Diets were 20% (w/w) fat (40% of calories) diets, high in either saturated fatty acids (lard-based) or polyunsaturated fatty acids (soybean oil-based) or standard laboratory chow (Purina, 4.5% (w/w) fat) given for 3 months. Rats that consumed the lard-based diet showed spatial memory impairment as tested by the delayed alternation radial arm maze task. The soybean oil-fed group was slightly impaired in the same task but consistently performed better than the lard-fed group. This study demonstrated that having high level of saturated fatty acids in the diet can impair learning and memory functions in rats (Greenwood and Winocur, 1990, 1996).

#### **1.7.2.2. Working memory**

Studies investigated the effect of a high fat diet on working memory produced different results. For example a diet composed of 60% fat and refined carbohydrates in Flinders Resistant Line rats reported no object recognition impairment (Abildgaard et al., 2011), however, in a study in 2008 more working memory errors in the radial arm maze observed by adult-aged (16-month old) rats that had cholesterol (2%) and saturated fat (hydrogenated coconut oil, Saturated Fat10%) diet (for 8 weeks) compared to normal chow group suggesting that a diet of saturated fat, hydrogenated fat and cholesterol can impair memory (Granholt et al., 2008). Moreover, a short term study demonstrated

that consumption of a high fat diet (55% kcal from fat) in rats for short term (9 days) impaired cognition including working memory as tested by the radial arm maze task (Murray et al., 2009).

## **1.8. Western and high fat diet, cognition and brain changes**

### **1.8.1. Acetylcholine**

Acetylcholine is an important and widely distributed neurotransmitter in the brain, involved in memory as well as regulation of cerebral blood flow (Schetinger et al., 1999). Acetylcholine is synthesised in presynaptic nerves from choline and acetylcoenzyme A (Acetyl CoA). Acetyl CoA and choline are produced as the result of the cell and lipid metabolism respectively. Acetylcholine appears to modulate learning and memory mainly through actions in the hippocampus and striatum (Pych et al., 2005). Research in human revealed that people who died of Alzheimer's disease had reduced number of cholinergic markers in their cerebral cortex (Whitehouse et al., 1982, Lyness et al., 2003) and this decrease in the number of cholinergic markers correlates with the degree of cognitive impairment (Bowen et al., 1976, Perry et al., 1978, Dournaud et al., 1995). Other studies showed that anti-cholinergic drugs may cause impairment in learning and memory, and drugs that support cholinergic function enhanced the memory of the animal models and patients that had Alzheimer's disease. Neurotransmitters synthesis can be affected by the diet as diet provides their precursors (Fernstrom, 1977, Anderson and Johnston, 1983). Moreover, several studies reported the effect of dietary changes on acetylcholinesterase level which is a hydrolase enzyme that breaks down acetylcholine by catalysing the hydrolysis of acetylcholine to choline and acetic acid. (Ruano et al., 2000, Vajreswari et al., 2002, Kaizer et al., 2004).

### **1.8.2. Glucose**

Glucose is the primary energy source of the brain and a fuel for most of its metabolic events. Glucose can easily enter the brain via glucose transporters that carry glucose across the blood-brain barrier. Glucose transporters in the central nervous system play an important role in neuronal activity, particularly cognitive function (Reagan et al., 2001). Moreover, glucose in the brain is the supply of precursor for several neurotransmitters including acetylcholine (Miccheli et al., 2003). Studies report positive effects on cognitive performance with glucose administration (Messier and Destrade, 1988, Means and Fernandez, 1992, Korol and Gold, 1998) and negative effects of long-term fasting due to eating disorders (Mathias and Kent, 1998). However, there appears to be no relation between lowered glucose levels as a result of missing a meal and cognitive performance, suggesting that cognitive changes are more complicated than simply being the result of transient, mild hypoglycaemia (Green et al., 1997).

Studies showed that the level of glucose transporter type 4 (GLUT4), which is an insulin-sensitive glucose transporter, in the hippocampus of obese rats was reduced relative to the control group. In another study, the memory of the obese Zucker rats were impaired as measured by hippocampus-dependent tasks (Winocur et al., 2005). These data suggest that glucose availability may impact memory formation.

#### **1.8.2.1. Glucose and acetylcholine**

Several pieces of evidence suggest that glucose interacts with cholinergic functions. Experiments revealed glucose attenuates amnesia in animals treated with scopolamine, a muscarinic cholinergic antagonist. (Stone et al., 1988, Stone et al., 1991). Another

series of experiments showed that glucose injections can attenuate the atropine-induced (Atropine is a muscarinic acetylcholine receptor antagonist) decrease of acetylcholine content in brain (Dolezal and Tucek, 1982), however, no effect was observed by glucose alone on acetylcholine content in animals (Dolezal and Tucek, 1982). Glucose injection, moreover, increased scopolamine-induced acetylcholine release from the hippocampus (Durkin et al., 1992). In another experiment, peripheral glucose injections could attenuate the depletion of acetylcholine content in the striatum and hippocampus (but not cortex) of rats treated with another muscarinic antagonist quinuclidinyl benzilate (Ricny et al., 1992). Based on the above studies, it has been suggested that glucose is taken into cholinergic presynaptic terminals and transformed into acetylcoenzyme A (the other acetylcholine precursor with choline) which is then used to synthesize acetylcholine (Messier and Gagnon, 1996).

### **1.8.3. Insulin-like hormones: Insulin and IGF-1**

Other factors that are influenced by the changes in diet and may have a role in memory performance are the family of Insulin-like growth factors. Those include insulin, IGF-1 (Insulin-like growth factor 1) and IGF-2 (Insulin-like growth factor 2), that are all polypeptide ligands (Efstratiadis 1998).

#### **1.8.3.1. Insulin**

Insulin is an adiposity signal; a peptide with 51 amino acids produced largely by pancreatic  $\beta$ -cells and its main well known role is regulation of blood glucose level (Duarte et al., 2012) as well as promoting glycogen and lipid synthesis and inhibiting lipolysis and fatty acid esterification (Wagle et al., 1975, Beynen et al., 1980, Baskin et al., 1999). Insulin acts on arcuate nucleus of hypothalamus and is important in energy

homeostasis by promoting glucose uptake using glial cells (Werner et al., 1989, Duarte et al., 2012). As well as regulation of food intake, insulin is important in neurotransmitter release and neuronal growth and survival in central nervous system (Schechter et al., 1998, Plitzko et al., 2001, Duarte et al., 2003, Gasparini and Xu, 2003, Duarte et al., 2004). Insulin can cross the blood brain barrier using an active transport mechanism (van Houten and Posner, 1979, Jialal et al., 1984, King and Johnson, 1985). Insulin receptors are widely distributed in the brain and have the highest concentrations in the olfactory bulb, hypothalamus, vertebral cortex, and hippocampus (Moll and Schubert, 2012). Animal and human studies revealed that brain insulin enhances memory (Kern et al., 1999, Park et al., 2000, Ghasemi et al., 2013) and this memory enhancing role of insulin is partly due to its positive effect on formation of synapses, synaptic function and synaptic plasticity (Schulinkamp et al., 2000, Kern et al., 2001, Banks, 2004, Benedict et al., 2007, Spielman et al., 2014).

Older adults with insulin resistance and rodents fed a high fat diet both showed cognitive impairment and responded to glucose consumption in a comparable manner (Greenwood and Winocur, 2005). In a study consumption of a high fat diet (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) by the Sprague Dawley rats for only 5 weeks, caused cognitive impairment in Morris water maze while the animals showed characteristic features of insulin resistance (Pathan et al., 2008).

#### **1.8.3.2. Insulin-like growth factor 1**

Insulin-like growth factor 1 (IGF-1) is a trophic factor with 70 amino acids that is mainly produced in the liver and is largely secreted when there is a high concentration of growth hormone. IGF-1 has different roles and mediates many functions of the

growth hormone in the peripheral and central nervous system such as stimulating cell survival, inhibiting apoptosis, inducing glucose uptake by muscle cells and breakdown of fat (Shemer et al., 1987, Jones and Clemmons, 1995, Rollero et al., 1998, Chen et al., 2000, De Meyts and Whittaker, 2002, Troncoso et al., 2012). IGF-1 is also important for the synthesis of neurotransmitters and their release (Anlar et al., 1999).

IGF-1 receptor and insulin receptor are parts of the receptor tyrosine kinase (RTK) family (Hubbard and Till, 2000). IGF-1 receptor is very similar to insulin receptor in organization and in sequence (varying from 41% to 84%) (Adams et al., 2000) and both consist of a membrane bound domain with tyrosine kinase activity that becomes activated when the insulin or IGF-1 binds to the receptor.

IGF-1 is widely distributed in the central nervous system and at high levels in the hippocampus (Sara and Carlsson-Skwirut, 1988, Kar et al., 1997). Studies demonstrated that IGF-1 is a key modulator of hippocampal neurogenesis and a major activator of the extracellular receptor kinase pathway that is central in learning and memory (Casadesus et al., 2004). Xiuqun Gong and colleagues showed that IGF-1 and IGF-1 receptor is down-regulated in the hippocampus of rats with vascular dementia (Gong et al., 2012). Lupien et al provided evidences on prevention of cognitive impairment in diabetic rats by systemic IGF-1 administration (Lupien et al., 2003), moreover, administration of exogenous IGF-1 reversed the impaired spatial learning in mutant mice with low levels of serum IGF- 1 (Trejo et al., 2008). Furthermore, other studies suggest that drugs, such as donepezil and cilostazol (selective AChE inhibitor) may improve cognitive function in mice by increasing the production of IGF-1 in the hippocampus (Narimatsu et al., 2009, Zhao et al., 2010). Evidence suggests that



obesity is closely associated with reduced levels of circulating growth hormone and IGF-1 (Spielman et al., 2014).

#### **1.8.3.3. Insulin-like growth factor 1 and acetylcholine**

Studies revealed that IGF-1 inhibits acetylcholine release from the hippocampus and cortex but not the striatum (Kar et al., 1997, Seto et al., 2002). Treatment with the selective AChE inhibitor, donepezil elevated basal IGF-1 markedly (Obermayr et al., 2005).

#### **1.8.3.4. Insulin-like growth factor 1 and neuronal activity**

During injury or as the result of environmental stimulation, IGF-1 is involved in the maintenance of neuronal activity and synaptic plasticity (Seto et al., 2002). Moreover, studies revealed that systematic injection of IGF-1 can mimic the effects of exercise in the brain (Carro et al., 2000). A same pattern of neuronal accumulation of IGF-1 and an identical widespread increase in neuronal c-fos (a marker of neuronal activity) were observed after exercise or intracarotid injection of IGF-1. Interestingly, by blocking the uptake of IGF-1 by brain cells, the exercise-induced increase in c-fos expression was also blocked (Carro et al., 2000).

#### **1.8.4. Neuronal activity**

C-fos is a protein which is the product of an immediate-early gene (IEG). Activation of c-fos gene is indicative of a change in neuronal activity. C-fos IEG is usually induced after stimuli such as learning (Kaczmarek, 1993, Herdegen and Leah, 1998, Radulovic et al., 1998, Santin et al., 2003). Research showed that obesity can affect basal

neuronal activity in specific areas of hypothalamus. Increased body weight was accompanied by an increase in basal neuronal c-fos-like immunoreactivity in the lateral hypothalamus, the lateral part of the dorsomedial hypothalamic and perifornical nuclei of diet-induced obese mice (Lin and Huang, 1999). Interestingly in another study neuronal activity in the ventromedial hypothalamic nucleus is decreased in mice exposed to a high fat diet but is increased in exercised mice independent of adiposity (Krawczewski Carhuatanta et al., 2011). The effect of obesity and a high fat diet on other areas of the brain need further investigation.

#### **1.8.5. Inflammation**

In response to harmful stimuli, the innate immune system of the body is activated and inflammation occurs as a natural response to the pathogens or any other stimuli. This inflammatory process is designed to fight the infection, signals the immune cells to move to the site of injury and starts the immune response (Davalos et al., 2005, Smith et al., 2012). This inflammatory process can occur in the periphery and/or in the brain.

It has been established that obesity is associated with peripheral inflammation. Interestingly, studies demonstrated that obesity is also associated with the inflammation in some areas of the brain. There are several potential mediators that could carry the inflammatory effects of a high fat diet to the brain (Pistell et al., 2010). Specific areas of the brain including the cortex and hippocampus that are important in memory and cognition have been shown to have the highest levels of cytokine binding (Parnet et al., 2002) and it has been previously shown that some of the cytokines such as IL-1 $\beta$  (Interleukin 1 $\beta$ ) and IL-6 (Interleukin 6) can disrupt neurophysiologic mechanisms involved in cognition and memory (Bellinger et al., 1995, Jankowsky and

Patterson, 1999, Gemma and Bickford, 2007). Bilbo and colleagues reported an increased level of IL-1 $\beta$  in the hippocampus of the obese pups that fed high fat diet compared to the lean animals (Bilbo and Tsang, 2010). In another study, diet-induced obese mice that have cerebral amyloid angiopathy (that is the building up of amyloid protein within the cerebrovasculature) showed an increased level of IL-6 in the striatum compared to healthy control mice (Zhang et al., 2013). Moreover, studies demonstrated that after an infection, the performance in the Morris water maze, which is an indicative of spatial memory is weakened (Barrientos et al., 2006, Sparkman et al., 2006, Sobesky et al., 2014).

#### **1.8.6. Astrocytes**

Astrocytes are the most abundant cells in the brain (Ridet et al., 1997, Moraga-Amaro et al., 2014) and have diverse roles in the central nervous system. Besides transporting nutrients within the brain, astrocytes express receptors for several neurotransmitters, neuropeptides and growth factors, produce neuroactive substances, and express key enzymes necessary for sensing and processing nutritional signals as well as maintenance of the blood brain barrier and supporting neurons physically and biochemically (Dehouck et al., 1990, Diano et al., 1998, Nedergaard et al., 2003, Garcia-Segura et al., 2008). Diet-induced obesity increases the astrocyte expression of leptin receptor in the hypothalamus (Hsuchou et al., 2009a) and it has been suggested that this increase specifically in a high fat fed animal contributes to obesity onset and perpetuation (Pan et al., 2008, Hsuchou et al., 2009a, Hsuchou et al., 2009b). Mice maintained on a high fat diet for 20-week study period experienced increase in GFAP-positive astrocytes in the arcuate nucleus of the hypothalamus (Berkseth et al., 2014). Further analysis of astrocyte morphology and density in the CA3 area of the

hippocampus revealed that in high fat fed mice, astrocytes developed longer and less abundant projections. These changes were accompanied by the up regulation of both glutamate transporter type-1 (GLT-1), an astrocyte glutamate transporter, suggesting that the learning and memory impairment observed in high fat fed mice is concomitant with morphological changes of astrocytes within the hippocampus (Cano et al., 2014).

### **1.8.7. Lipids: Cholesterol and Triglyceride**

Studies that have investigated the role of serum cholesterol in brain functions independent of vascular effects demonstrated mixed results, with some noting a correlation between lipid levels and cognitive function, whereas other studies did not (Rogers et al., 1989, Reitz et al., 2005). There is compelling evidence that triglycerides and/or free fatty acids are able to alter the function of central nervous system (Farr et al., 2008). This is further supported by clinical studies that show elevated triglycerides in association with poor cognitive performance in patients with type 2 diabetes (Perlmutter et al., 1988). Other potential means by which triglycerides may adversely affect the brain is through their breakdown into free fatty acids, which can also be formed and released directly from adipocytes. It has been also suggested that triglycerides may impair cognition by preventing leptin from reaching the brain regions important for learning and memory (Banks et al., 2004).

## **1.9. Hypothesis and aims**

I hypothesised that a western and a high fat diet cause multiple changes in those areas of the brain that are involved in memory regulation and affect performance in memory tasks.

The aims of this research were to investigate the changes that occur in brain areas important for memory regulation resulting from consumption of a higher percentage of fat in diet. More specifically, this project investigates:

- 1) The changes that occur in the level of acetylcholine in medial septum and vertical limb of the diagonal band (MS/VDBD), CA1, CA2&3 and dentate gyrus in the hippocampus and striatum of rats following consumption of a high fat and a western diet.
- 2) The changes that occur in neuronal activity in CA1, CA2&3 and dentate gyrus regions of the hippocampus, retrosplenial granular a and b cortex, infralimbic, prelimbic and anterior cingulate of prefrontal cortex, nucleus accumbens, paraventricular nucleus and arcuate nucleus of the hypothalamus and paraventricular nucleus of thalamus following consumption of a western diet in rats.
- 3) The changes that occur in the number of astrocytes in CA1, CA2&3 and dentate gyrus regions of the hippocampus and infralimbic, prelimbic and anterior cingulate of prefrontal cortex
- 4) The changes that occur in the number of IGF-1 in infralimbic, prelimbic and anterior cingulate of prefrontal cortex following consumption of a western diet in rats.
- 5) The effect of a high fat and a western diet on spatial and working memory paradigms in rats.

## **2. Chapter 2: Effect of a western and a high fat diet on memory and cholinergic measures in rats.**

### **2.1. Introduction**

Recent evidence reports a fast increase in the incidence of obesity (World Health Organization, 2015). The major increase in the consumption of fast food and high fat diet contributed significantly to this increase (Polk, 2005) to the extent that currently more than one-third of the population are overweight (World Health Organization, 2015). This rise caused overweight and obesity to be announced as the fifth leading risk for death worldwide (World Health Organization, 2015) leading to many other complications such as type 2 diabetes, insulin resistance and cardiovascular disease (Swinburn, 1993, Wilson et al., 2002, Mokdad et al., 2003, Li et al., 2005, Lavie et al., 2014). Moreover, obesity created enormous burden in the healthcare budget (Lehnert et al., 2013). Studies revealed an association between obesity and cognitive decline in humans (explained in 1.2.1.2.) (Elias et al., 2003, 2005, Waldstein and Katzel, 2006, Farr et al., 2008) and in rodents (explained in 1.7) (McNeilly et al., 2011, Valladolid-Acebes et al., 2011). However, the changes that occur in brain as the result of being overweight and obese and the types of memories and the areas of the brain that are affected by obesity, are still unknown.

Finding an animal model that can be used to investigate those changes is the first challenge. As increase in dietary fat intake is one of the main factors responsible for the increase in the incidence of obesity in human, therefore among the various obesity animal models (explained in 1.3.), using the animal models that develop obesity as the result of consuming higher percentage of fat in diet is elucidative and this will assist to investigate the changes that occur in the brain as the result of diet (explained in 1.8.)

and it may assist to explore how dietary changes affect memory. However, it has been shown that there are differences between and within the strains of animals in developing obesity as the result of higher percentage of fat in diet (Levin et al., 1997, Farley et al., 2003). Moreover, other factors play a role in the effect of diet on cognition such as the age of the animal at the start of the diet change (Privitera et al., 2011) and the duration of feeding (Murray et al., 2009). More importantly, the composition of diet including the percentage of fat and cholesterol has a critical role in its effect on body weight and can cause different complications (Rothwell et al., 1982, Buettner et al., 2006).

In rodents in various studies, higher percentage of fat in diet is conducive to obesity and increased level of epididymal fat pad. For example, our laboratory showed that 36% fat diet for 12 weeks in male Wistar rats can cause a significant increase in epididymal fat pad and increase in fasting blood glucose without changing the body weight, compared to normal diet (Chen et al., 2010). In my first study, I placed rats on a high fat diet or western diet for three months in order to investigate the changes and the differences that occur in behaviour and brain as the result of higher percentage of fat in diet. Western diet (WD SF00-219, 21% total fat including 1.80% total saturated fats and 0.15% cholesterol) is designed to mimic the western fast food diet and its formula is equivalent to Harlan Teklad TD88137 or Research Diets Western Diet D12079B that has been used extensively in different studies (Plump et al., 1992, Tsuchiya et al., 2012) and SF02-006 with 60% fat was used to further investigate this effect. These diets could cause an increase in body weight and blood pressure, and leads to high cholesterol and triglyceride levels as previously reported (Dhar et al., 2000) (details of the diets are available in the methods 2.2.1).

Different memory paradigms such as Y-maze and novel object recognition task can be used in order to investigate what type of memory may get affected as the result of the higher percentage of fat in diet.

### **2.1.1. Y-maze**

The tendency of rodents to alternate their choice of arms in Y-maze without any reinforcement was first introduced by Tolman (Tolman, 1925). Based on this spontaneous inclination to explore novel places and objects (Dember and Fowler, 1958, Poucet et al., 1986, Crusio et al., 1989) without using any motivator, Y-maze is designed and developed to test different aspects of spatial memory (Gerlai, 1998). In the Y-maze alternation task, the rodent is placed in the maze to explore it, where the entry to one of the arms is blocked. After a specific interval, the animal is put into the maze again to explore all three arms of the maze. This paradigm is based on the principle that rodents with the assistance of the spatial clues around the arm form a memory of the arms they visit. During the test phase, rodents will recall the previously visited arms and prefer to choose the novel arm. The number of entries into the novel arm and the time the animal spends in the novel arm are used for analysis of the result (Douglas, 1966, Gerlai, 1998). In order to investigate the effect of diet on spatial and working memory, Y-maze and novel object recognition paradigms were used.

### **2.1.2. Novel object recognition task**

Novel object recognition occurs as a result of spontaneous exploration by rodents. It was first studied and developed by Ennaceur and Delacour as a measure for memory function (Ennaceur and Delacour, 1988, Ennaceur and Meliani, 1992). While the details of the novel object recognition test varies between studies, there are two distinct



phases in this task. During the first phase, rodents are exposed to two identical objects, followed by an inter-trial interval. In the second phase, rodents are exposed to one of the previously investigated (familiar) objects and a new object. Objects should differ in terms of their shape, colour and texture (Ennaceur et al., 2009). Normal rodents store a memory of the familiar object, recall it during the second phase and discriminate between the two objects and as a result, spend more time exploring the novel object (Ennaceur and Delacour, 1988). The differences in exploration time of the novel and the familiar object in the second phase are used as a measure for recognition in rodents including the time the rodent spent exploring the novel object or calculating the discrimination ratio  $[(\text{time spent exploring the novel object} - \text{time spent exploring familiar object}) / \text{time spent exploring both objects}]$  (Ennaceur et al., 1997). Studies in rodents demonstrated that perirhinal cortex is an important brain region in this task (Aggleton et al., 1997) and the medial prefrontal cortex has an important role in recognition of the sequence of presented object in this paradigm (Ennaceur et al., 1997, Mitchell and Laiacina, 1998).

This chapter aims to investigate the effect of consuming a western and a high fat diet on brain pathology specifically by analysing the changes in the acetylcholine level in different areas of the brain. As mentioned in 1.8.1, acetylcholine is a neurotransmitter that has an important role in learning and memory specifically in the hippocampus and striatum. Acetylcholine is synthesized in the nerve endings of the presynaptic nerve from choline and acetyl coenzyme A. The changes in acetylcholine level have been analysed by measuring the level of choline acetyltransferase and acetylcholinesterase. Choline acetyltransferase (ChAT) is an enzyme expressed in presynaptic terminals of neurons and it is responsible for production of acetylcholine, by catalysing the transfer of the acetyl group of acetyl CoA to choline. Acetylcholinesterase is a hydrolase

enzyme in the central nervous system that breaks down acetylcholine by catalysing the hydrolysis of acetylcholine to choline and acetic acid. Moreover, acetylcholinesterase has an important role in regulation of the transmission of nerve impulses across cholinergic synapses (Szegeletes et al., 1999). The abundance of acetylcholinesterase is a good indicator of cholinergic activity (Schetinger et al., 1999). Previous studies demonstrated that changes in diet such as variation in glucose (Olivier et al., 2001) or higher percentage of sugar and fat together for six month (Kaizer et al., 2004) can affect cholinesterase activities. Further investigation is required to elucidate the effect of higher percentage of fat in diet of adult rats on their cholinergic activity in specific brain areas.

The hippocampus, as a part of the limbic system that is enriched with cholinergic innervation, has a critical role in cognitive functions. Anatomical and physiological data indicate that acetylcholine regulates the activity of cholinergic neurons (Hasselmo, 2006). In this chapter, I investigated the effect of higher percentage of fat in diet in the hippocampus as well as the medial septum (MS) and vertical limb of the diagonal band (VDBD) in the forebrain since the cholinergic input from those areas is increased from primary sensory areas of cortex to cortical regions that target the hippocampus (Gaykema et al., 1991, Dutar et al., 1995, Gallagher and Colombo, 1995). Moreover, these areas are more vulnerable to changes such as aging or Alzheimer's disease (Dutar et al., 1995). The CA1 (cornu ammonis 1) and DG (dentate gyrus) in dorsal hippocampus receive input from VDBD and cells in ventral hippocampus are innervated by both VDBD and medial septum (Nyakas et al., 1987).

## **2.2. Materials and methods**

### **2.2.1. Animals and chow**

Male Long Evans rats (Monash University, Australia) weighing 240 - 292g at the start of feeding were housed in groups of three under a light/dark cycle (12 hour/12 hour), in a temperature controlled room (22°C) with food and water *ad libitum*. Animals were randomly divided into three groups (n=12) and assigned to either a control (Con; Standard AIN93G rodent diet, 7% total fat including 1.05% total saturated fatty acids; Specialty Feeds, Perth, Australia), western diet (WD; SF00-219, 21% total fat including 1.80% total saturated fats and 0.15% cholesterol; Specialty Feeds, Perth, Australia) or a high fat diet (HF; SF02-006, 60% fat modification of AIN93G including 1.10% total saturated fats; Specialty Feeds, Perth, Australia). Table 1 outlines the details of the three diets used according to the provider company.

Animals received the diets for 12 weeks after which behavioural testing commenced. Food intake and body weight were measured twice-weekly for three months. From this, caloric intake was calculated. All experimental protocols were performed in accordance with the prevention of Cruelty to Animals Act 1986 (Australia), the National Health and Medical Research Council (NHMRC) code EA28 and approved by the RMIT University Animals Ethics Committee.

Analysis of Diet	AIN-93G	SF00-219	SF02-006
	Control Diet	Western Diet	High fat Diet
Protein	18.7%	19.00%	19.40%
Fat	7.0%	21.00%	60.00%
Total Saturated Fatty Acids	1.05%	1.80%	1.10%
Total n3 Fatty Acid	0.55%	0.35%	1.39%
Total n6 Fatty Acid	Not available	0.41%	3.41%
Fibre	5.0%	-	-
Crude Fibre	-	4.70%	4.70%
% Total calculated digestible energy from lipids	16.4%	40.00%	81.00%
% Total calculated digestible energy from protein	18.8%	17.00%	13.00%

**Table 2.1 Diet composition of the control (AIN-93G), western (SF00-219) and high fat (SF02-006) diets**

### **2.2.2. Blood pressure measurement**

Systolic blood pressure was monitored weekly in conscious resting animals by non-invasive tail-cuff plethysmography (NIBP controller on Powerlab, AD Instruments, Colorado Springs, USA).

### **2.2.3. Behavioural testing**

After twelve weeks of feeding the control, western and high fat diet, the Novel object recognition task and Y-maze paradigm were performed as described below.

#### **2.2.3.1. Novel object recognition test**

Apparatus consisted of a black wooden box (60 cm long x 60 cm wide x 50 cm high), with a DVD camcorder (Canon, Japan) positioned directly above in order to record behaviour. Objects to be discriminated were glass or ceramic and were approximately equal in size and heavy enough not to be displaced by the animals. Rats were habituated to the box twice for 3 minutes each to ensure sufficient familiarity with the testing environment. On the test day, each rat was placed in the box and allowed to explore two identical objects for a period of 3 minutes (trial phase). The rat was then returned to its home cage for a 1 hour inter-trial interval. The box and objects were cleaned with 70% ethanol. In the test phase, the rat was returned to the box and allowed to explore a familiar and a novel object for a further 3 minutes. Familiar and novel objects were changed between left and right position to prevent location bias as in Figure 2.1. Both trial and test phases were filmed and recorded for subsequent behavioural analysis. Object exploration was defined as the rats sniffing or touching the objects but not by leaning against, standing on, turning around or sitting on the

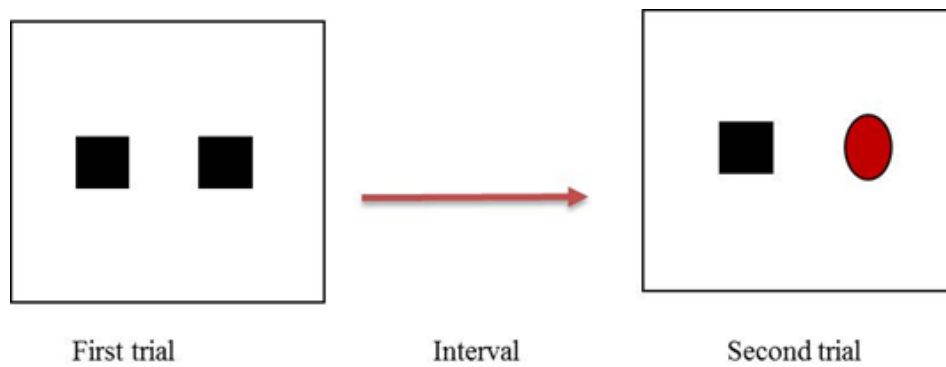
objects. The exploration time (s) for each object was recorded manually. Discrimination ratio (DR) [(time spent exploring the novel object – time spent exploring the familiar object)/total exploration time] was then calculated for each of the animals during the test phase.

#### **2.2.3.2. Y-maze**

The Y-maze was a three-arm maze with equal angles between all arms (50cm long x 17cm wide x 32cm high). Rats were habituated twice to the maze, each time for 5 minutes to become familiar with the testing environment. On the test day, rats were allowed to explore the maze for 10 minutes while having access to two of the three arms (home, or start arm, and familiar arm). The rat was then returned to its home cage for a 4 h inter-trial interval during which the maze was cleaned with 70% ethanol. The rat was then again placed back into the maze, this time having access to all arms for 5 minutes. Both trial and test phases were filmed and recorded for subsequent behavioural analysis. The number of entries to the novel arm and the time rat spent in each arm was recorded manually by stopwatch.

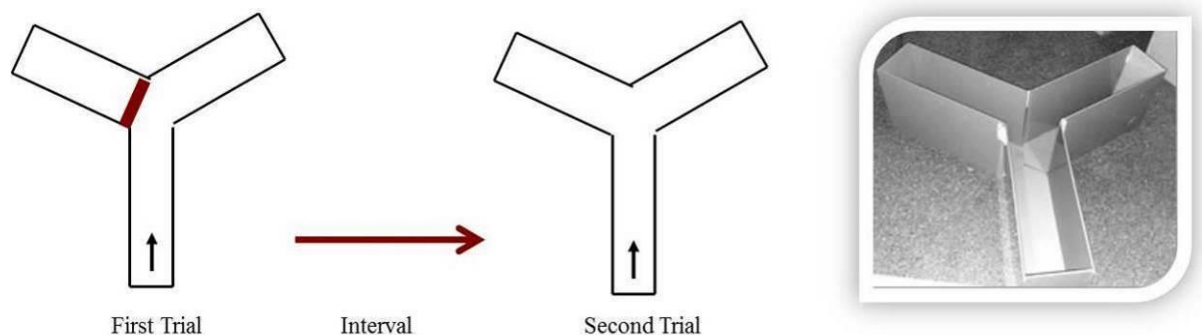
#### **2.2.4. Glucose tolerance test**

At the end of the study, rats were fasted overnight, and a glucose tolerance test (GTT) performed. Blood samples were collected from a single wound made by cutting the tip of the tail. Blood glucose levels were measured before and at 5, 10, 20, 30, 45, 60, 90, and 120 minutes after an intraperitoneal injection of D-glucose (2 g/kg of a 0.5 g/ml solution). Blood glucose levels were tested using a commercial glucose testing kit (ACCU-CHEK, Roche Diagnostics, Germany). Data was expressed as the area under the curve of the 2 hour test.



**Figure 2.1 Schematic diagrams of the novel object recognition paradigm;**

This consists of a black wooden box (60 cm long x 60 cm wide x 50 cm high), with glass or ceramic objects. Each rat is placed in the box and allowed to explore two identical objects for a period of 3 minutes. The rat is then returned to its home cage for a 1 hour inter-trial interval. In the test phase, the rat is returned to the box and allowed to explore a familiar and a novel object for a further 3 minutes.



**Figure 2.2 Schematic diagrams of the Y-maze paradigm;**

The Y-maze consists of a three-arm maze with equal angles between all arms (50cm long x 17cm wide x 32cm high). Rats are allowed to explore the maze for 10 minutes while having access to two of the three arms (home, or start arm, and familiar arm). The rat is then returned to its home cage for a 4 h inter-trial. The rat is then again placed back into the maze, this time having access to all arms for 5 minutes (second trial).

### **2.2.5. Collection of blood and brain**

Rats received a lethal dose of sodium pentobarbital (100mg/kg body weight, i.p.). Trunk blood was collected, centrifuged at 3000 RPM for 20 minutes at 6°C and the serum transferred to -20°C. Brains were removed and placed into 4% paraformaldehyde in phosphate buffer before being transferred to a 20% sucrose solution prior to sectioning. Coronal sections (30µm) thickness were cut on a cryostat (Leica CM 1900 Microsystems Nussloch GmbH, Germany) and stored in cryoprotectant (37.5% sucrose, 1.25% polyvinyl pyrrolidone, 37.5% ethylene glycol in 0.1M phosphate buffered saline (PBS)) in 24 well plates until required for analysis.

### **2.2.6. Choline acetyltransferase immunohistochemistry**

Three sections from medial septum/vertical limb of the diagonal band (MS/VDBD) (Bregma +0.2 and +0.7) of each animal (determined using a rat brain atlas) were analysed for choline acetyltransferase (ChAT). Sections were washed in PBS, then blocked for 45 minutes with 3% Normal Rabbit Serum (PBS, 0.1% TritonX and 1% Bovine Serum Albumin), before being incubated for 48 hours with anti-ChAT antibody (Chemicon, 1:500). After washing, sections were incubated for 1 hour with Rabbit anti-goat biotinylated antibody (Vector, 1:200) and processed by the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, UK). Peroxidase was visualised using 3,3-diaminobenzadine (DAB) intensified with nickel chloride. Slides were air dried, dehydrated in ascending concentrations of ethanol followed by xylene, and cover-slipped with Depex mounting medium.



### **2.2.7. Acetylcholinesterase staining**

Six sections from each animal were used in order to analyse hippocampus (CA1, CA2&3 (Cornu Ammonis 2&3), dentate gyrus) and striatum. Briefly, slides were washed in PBS then incubated overnight in an enzymatic solution containing 0.005% ethopropazine, 0.09% glycine, 0.06% cupric sulphate pentahydrate, 0.12% acetylthiocholine iodide and 1.78% acetate acid, pH 5.0. Slides were then rinsed in distilled water, developed for 2 minutes in a solution of 0.70% sodium sulfide at pH 7.3, rinsed, air-dried, dehydrated in increasing gradient of ethanols followed by xylene, and cover-slipped using Depex mounting medium.

### **2.2.8. Image analysis**

Six images were captured from each region of interest using an Olympus BX60 microscope (Japan) and RTKE Diagnostic Instruments SPOT camera (USA) connected to a PC computer with SPOT imaging software (USA). Counts of ChAT stained nuclei in the MS/VDBD were performed using the public domain Image J (USA) software. Densitometry measurements were performed using the public domain Scion Image Software (USA) program to quantify the optical density of acetylcholinesterase in the striatal and hippocampal subregions. For hippocampal subfields: dentate gyrus, CA1, CA2, CA3, and the striatum, the entire extent of the target region within the selected coronal sections were assessed. Wherever possible, counting procedures were done without knowledge of the group assignments.

### **2.2.9. Lipid measurements**

Serum lipids were measured with commercial kits (Triglyceride E and Cholesterol E test, Wako Pure Chemical Industries, Ltd., Japan).

### **2.2.10. Statistical analysis**

All data were analysed using SPSS for Windows Version 18.0. Body weight, food intake, calorie intake and blood pressure were analysed using two-way ANOVA with group and week as factors; when appropriate, simple effects for each group were analysed. Cholesterol, triglycerides, fasting blood glucose and glucose tolerance values were assessed by one-way ANOVA. Exploration times for the novel object recognition test were analysed using two-way ANOVA with group and object as factors; while discrimination ratios were evaluated by one-way ANOVA. To assess the performance in the Y-maze paradigm, time and entries to the novel arm were determined by one-way ANOVA with post hoc analysis. Brain neurochemistry was analysed using two-way ANOVA with group and region as factors; simple effects for each group were determined.

## **2.3. Results**

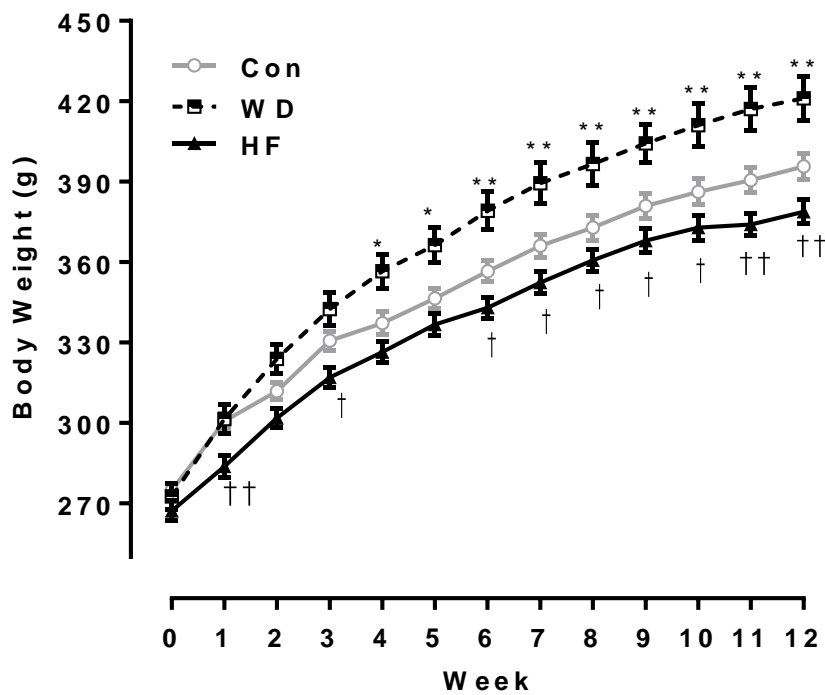
### **2.3.1. Effect of diet on body weight, percentage of initial body weight, food and calorie intake**

Body weights at the beginning of the dietary feeding regimen were not significantly different among the three groups (Con 267.6 g  $\pm$  2.3 g, WD 265.5 g  $\pm$  4.7 g, HF 267.1 g  $\pm$  3.8 g; (F(2,35)=0.09; p=0.92)). Analysis of the body weight over the 12 experimental weeks demonstrated a significant difference between the three groups

(Group:  $F(2,33)=10.0$ ,  $p<0.001$ ; Week:  $F(1,33)=1814.9$ ,  $p<0.001$ ; Group x week:  $F(2,33)=11.4$ ;  $p<0.001$ ). Post hoc analysis demonstrated that rats fed a western diet gained significantly higher body weight compared with the control diet from week 4. In contrast, rats fed the high fat diet had a significantly lower body weight compared to control for the second half of the feeding regime. The analysis of the percentage of the initial body weight revealed a significant difference between the groups (Group:  $F(2,33)=19.20$ ,  $p<0.001$ ; Week:  $F(1,33)=1468.725$ ,  $p<0.001$ ; Group x week:  $F(2,33)=6.97$ ,  $p<0.01$ ), however, post hoc analysis revealed no difference between the high fat diet and control group.

Average food intake over the entire experiment was significantly different between groups (Group:  $F(2,33)=168.7$ ,  $p<0.001$ ; Week:  $F(1,33)=101.6$ ,  $p<0.001$ ; Group x week:  $F(2,33)=64.7$ ,  $p<0.001$ ). Average weekly intake per rat was Con:  $15.7\text{g}\pm0.7\text{g}$ , WD:  $15.8\text{g}\pm0.6\text{g}$ , HF:  $12.6\text{g}\pm0.2\text{g}$ . Simple effect analysis showed that, this difference was due to a lower food intake of western diet ( $p<0.05$  weeks 1, 3-6) and high fat diet groups ( $p<0.05$  all weeks except week 11) compared with the control group.

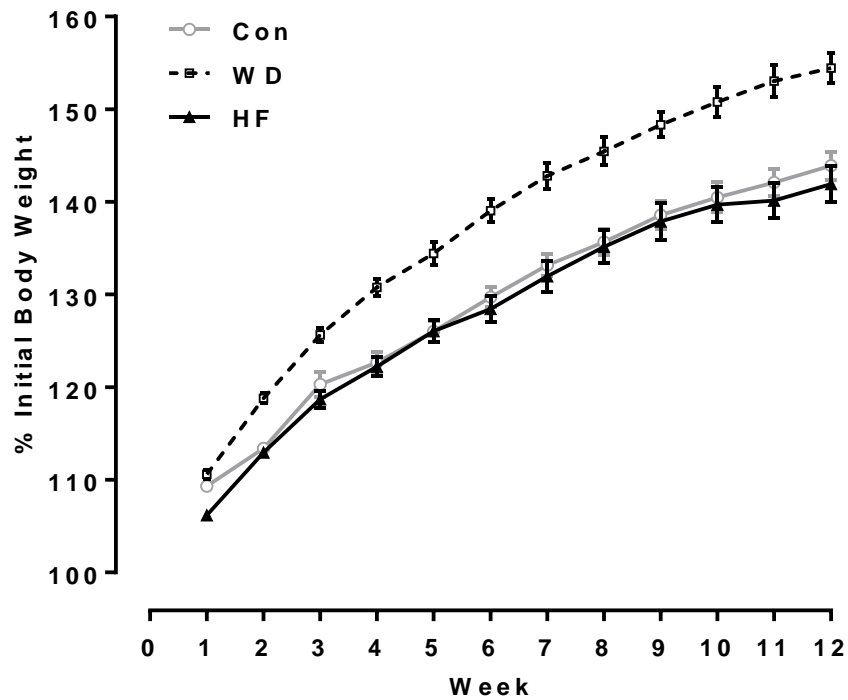
Caloric intake of the three groups were significantly different (Group:  $F(2,33)=52.4$ ,  $p<0.001$ ; Week:  $F(1,33)=279.7$ ,  $p<0.001$ ; Group x week:  $F(2,33)=26.4$ ,  $p<0.001$ ). Simple effect analysis demonstrated a significant exacerbation in calorie intake by rats who received a western and a high fat diet compared with control.



**Figure 2.3 Body weight (g) of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

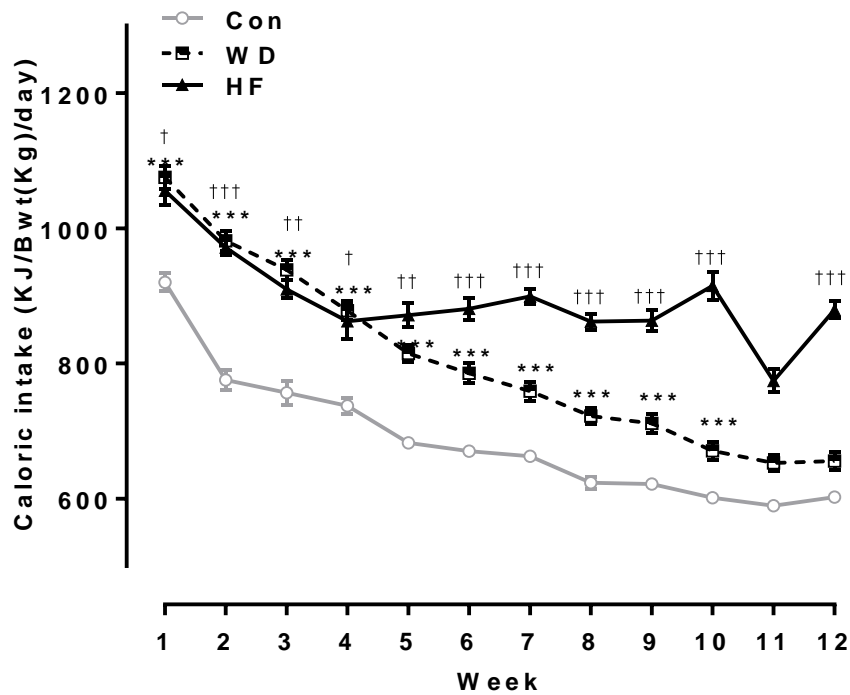
Rats fed a western diet gained significantly higher body weight compared with the control diet from week 4. Rats fed the high fat diet had a significantly lower body weight compared to control for the second half of the feeding regime. n=12.

\*p<0.05; \*\*p<0.01. Values are expressed as mean + SE.



**Figure 2.4 Percentage of initial body weight of rats fed control (Con), western diet (WD) or high fat diet (HF) for 12 weeks;**

A significant difference was observed between the three groups in the percentage of initial body weight (Group:  $F(2,33)=19.20$ ,  $p<0.001$ ; Week:  $F(1,33)=1468.725$ ,  $p<0.001$ ; Group x week:  $F(2,33)=6.97$ ,  $p<0.01$ ). No difference was observed between the high fat diet and control group.  $n=12$ . Values are expressed as mean + SE.



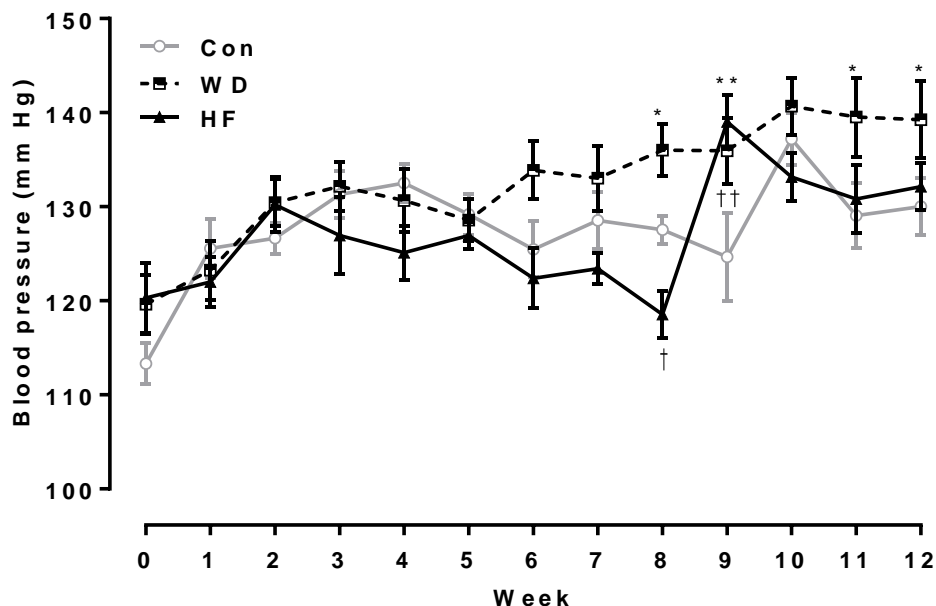
**Figure 2.5 Caloric intake (KJ/Body weight(Kg)/day) of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

Caloric intake of the three groups were significantly different (Group:  $F(2,33)=52.4$ ,  $p<0.001$ ; Week:  $F(1,33)=279.7$ ,  $p<0.001$ ; Group x week:  $F(2,33)=26.4$ ,  $p<0.001$ ). There was a significant exacerbation in calorie intake by rats that received a western and a high fat diet compared with control.  $n=12$ .

\* $p<0.05$ , \*\* $p<0.01$ ; \*\*\* $p<0.001$ . Values are expressed as mean  $\pm$  SE.

### 2.3.2. Effect of diet on blood pressure

Blood pressure was measured weekly in all rats (Group:  $F(2,33)=4.81$ ,  $p<0.05$ ; Week:  $F(1,33)=45.38$ ,  $p<0.001$ ; Group x week effect:  $F(2,33)=2.59$ ,  $P=0.09$ ). Post hoc analysis demonstrated that consumption of a western diet increased blood pressure compared with the control group toward the final weeks of the study (weeks 8, 10, 11 and 12:  $p<0.05$ ) (Figure 2.6). The effect of a high fat diet on blood pressure was variable: a significant decrease compared to the control group was measured at week 8 ( $p<0.05$ ), while a significant increase above control group was observed at week 9 ( $p<0.001$ ).



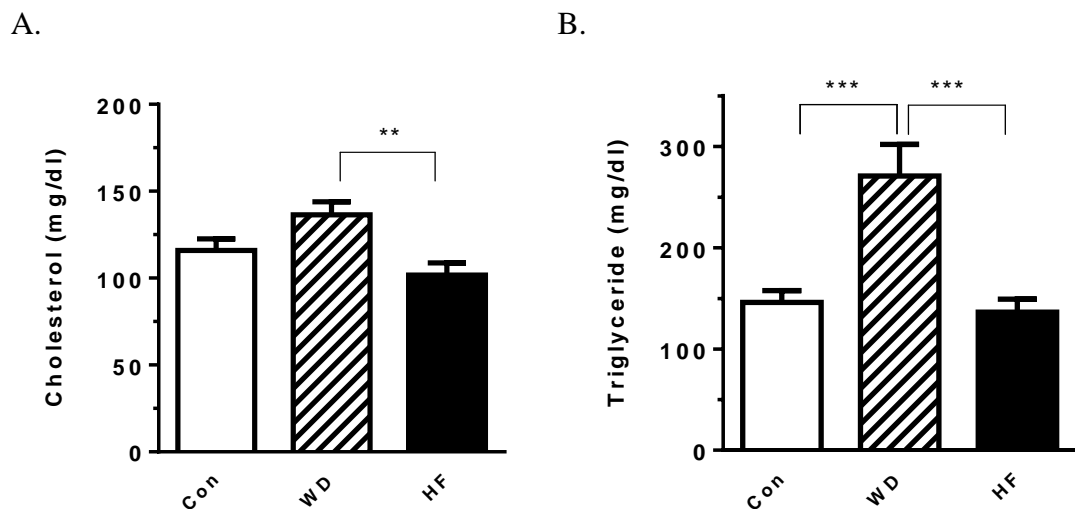
**Figure 2.6 Blood pressure (mm Hg) of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

Consumption of a western diet increased blood pressure compared with the control group toward the final weeks of the study (weeks 8, 10, 11 and 12). The effect of a high fat diet on blood pressure was variable with a significant decrease compared to the control group at week 8, and a significant increase above the control group at week 9.  $n=12$ . \* $p<0.05$ ; \*\* $p<0.01$ . Values are expressed as mean  $\pm$  SE.

### 2.3.3. Effect of diet on blood cholesterol and triglycerides

A significant difference was observed in the level of triglycerides among the three groups (Con  $145.9 \pm 11.6$  mg/dL, WD  $270.7 \pm 31.3$  mg/dL, HF  $134.5 \pm 13.0$  mg/dL; ( $F(2,33)=13.14$ ,  $p<0.001$ )). This reflected a higher level of triglyceride in a western diet compared with control or high fat groups (both  $p<0.001$ ).

Blood cholesterol levels were significantly different among the groups (Con  $115.9 \pm 6.7$  mg/dL, WD  $136.5 \pm 7.5$  mg/dL, HF;  $101.8 \pm 7.0$  mg/dL; ( $F(2,33)=6.09$ ,  $p<0.01$ )). This difference was reflected in the higher cholesterol level of the western diet group compared with high fat ( $p<0.01$ ) but not the control group ( $p=0.14$ ).



**Figure 2.7 Serum total cholesterol (mg/dl) and Serum triglyceride (mg/dl) after 12 weeks of control (Con), western diet (WD) and high fat diet (HF) consumption;**

A. Blood cholesterol level was higher in the western diet group compared with the high fat but not the control group. B. A higher level of triglyceride was observed in the western diet compared with the control or high fat diet groups.  $n=12$ . \*\* $p<0.01$ ; \*\*\* $p<0.001$ . Values are expressed as mean  $\pm$  SE.



#### **2.3.4. Effect of diet on fasting blood glucose and glucose tolerance**

Fasting blood glucose levels were found to be different among the groups (Con  $9.7 \pm 0.2$  mmol/L, WD  $10.2 \pm 0.3$  mmol/L, HF  $9.1 \pm 0.2$  mmol/L; ( $F(2,33)=4.67$ ,  $p<0.05$ ). This reflected a significant difference between the western diet and the high fat diet groups ( $p<0.05$ ), while the level of fasting blood glucose was not significantly different in rats fed the high fat ( $p=0.24$ ) or western diet ( $p=0.23$ ) compared to the control group. This difference was not transferred to glucose tolerance (area under the curve, Con  $1024.8 \pm 43.1$ , WD  $949.2 \pm 31.5$ , HF  $947.7 \pm 37.3$ ; ( $F(2,35)=0.75$ ,  $p=0.48$ ) .

#### **2.3.5. Effect of diet on working memory**

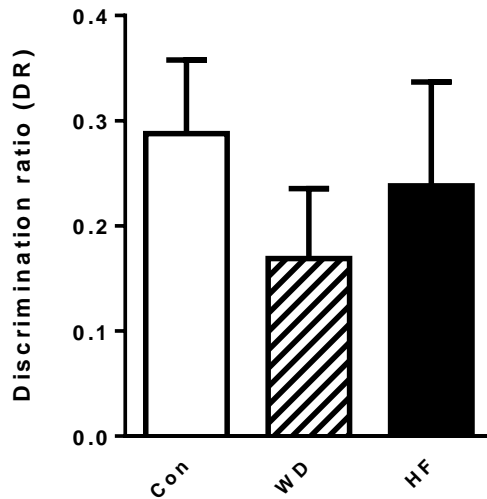
In the novel object recognition test, all rats spent equivalent time exploring the identical objects (left and right) in the trial phase (left object: ( $F(2,35)=0.8$ ,  $p=0.47$ ); right object: ( $F(2,35)=1.3$ ,  $p=0.30$ ); total: ( $F(2,35)=0.1$ ,  $p=0.90$ )) as shown in table 2.2. In the test phase, all rats spent more time exploring the novel object, with no significant differences in exploration times between groups (novel object: ( $F(2,35)=0.52$ ,  $p=0.60$ ); familiar object: ( $F(2,35)=0.85$ ,  $p=0.44$ )) as demonstrated in Figure 2.8. As such there was no observed change in discrimination ratio ( $F(2,35)=0.56$ ,  $p=0.58$ ) (Figure 2.8).

<b>Trial</b>	<b>Left object (s)</b>	<b>Right object (s)</b>	<b>Total (s)</b>
<b>Con</b>	15.1±1.6	14.0±1.9	29.1±3.1
<b>WD</b>	12.8±1.0	18.0±2.3	30.8±2.9
<b>HF</b>	15.2±1.8	14.0±2.0	29.2±3.2

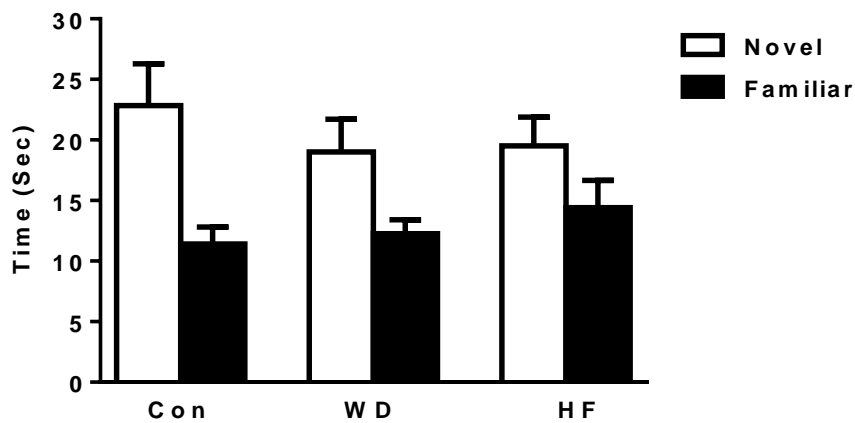
**Table 2.2 Novel objection recognition results. Time exploring objects (seconds, s) in the trial phase by rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

All rats spent equivalent time exploring the identical objects (left and right) in the trial phase (left object: (F(2,35)=0.8, p=0.47); right object: (F(2,35)=1.3, p=0.30); total: (F(2,35)=0.1, p=0.90)) n=12. Values are expressed as mean  $\pm$  SE.

A.



B.

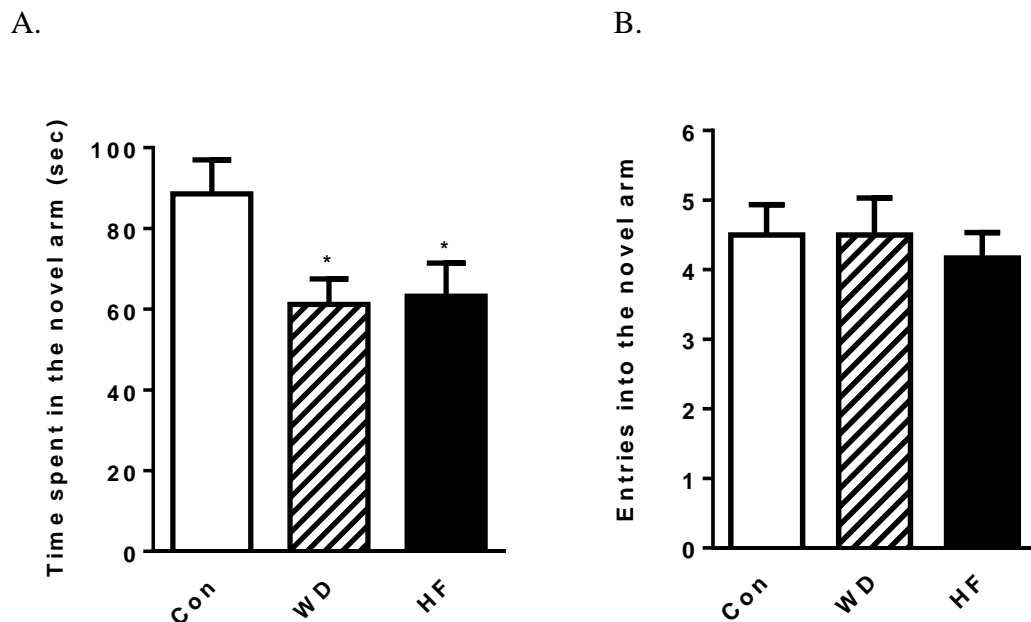


**Figure 2.8 Novel objection recognition results of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

A. Time exploring familiar and novel objects in the test phase. All rats spent more time exploring the novel object, with no significant differences in exploration times between the groups (novel object: ( $F(2,35)=0.52$ ,  $p=0.60$ ); familiar object: ( $F(2,35)=0.85$ ,  $p=0.44$ ))  $n=12$ . B. Discrimination ratio scores from test phase. There was no observed change in discrimination ratio ( $F(2,35)=0.56$ ,  $p=0.58$ )  $n=12$ . Values are mean  $\pm$  SE.

### 2.3.6. Effect of diet on spatial memory

Investigating the time spent in the novel arm of the Y-maze, revealed a significant difference among the groups ( $F(2,34)=3.94$ ,  $p<0.05$ ). Further analysis demonstrated that both the western diet and the high fat diet groups spent significantly less time in the novel arm ( $p<0.05$ ) compared with the control group (Figure 2.9). Number of novel arm entries was not significantly different among the three groups ( $F(2,34)=0.14$ ,  $p=0.87$ ) (Figure 2.9).

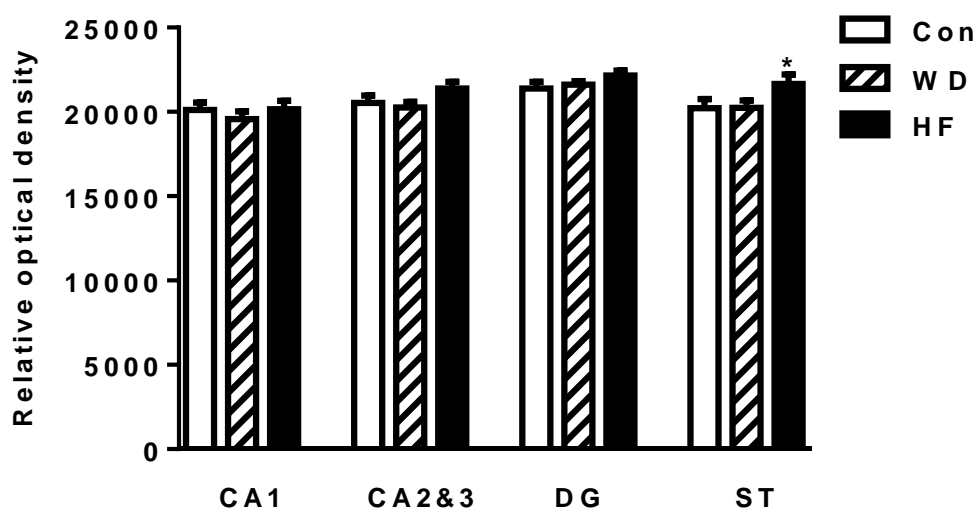


**Figure 2.9 Y-maze data of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;**

A. Time exploring novel arm in the test phase. Both the western diet and the high fat diet groups spent significantly less time in the novel arm compared with the control group  $n=12$ . Values are expressed as mean  $\pm$  SE. \* $p<0.05$ . B. Number of entries into the novel arm in the test phase was not significantly different among the three groups.  $n=12$ . Values are expressed as mean  $\pm$  SE.

### **2.3.7. Effect of diet on cholinergic activity**

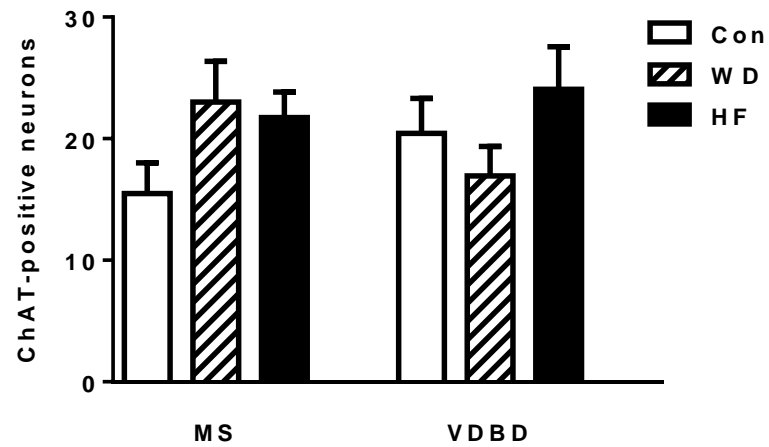
Investigating the effect of diets on different brain areas showed a significant difference in acetylcholine esterase activity among the groups ( $F(1,27)=3.5$ ,  $p<0.05$ ), (CA1; Con and WD  $n=12$ , HF  $n=9$ , CA2&3; Con and WD  $n=12$ , HF  $n=8$ , DG; Con and WD  $n=12$ , HF  $n=10$ , ST; Con= $12$ , WD and HF  $n=10$ ). This reflected a significant increase in acetylcholine esterase activity in the striatum of the rats after a high fat diet compared with the control group ( $p<0.05$ ). No change was observed in CA1, CA2&3 or dentate gyrus after a western diet or a high fat diet (Figure 2.10). Number of ChAT-positive cells was measured within MS and VDBD. No differences were observed in MS ( $F(2,33)=2.4$ ;  $p=0.11$ ), (Con and HF group  $n=12$  and WD  $n=10$ ) or VDBD area ( $F(2,33)=1.4$ ,  $p=0.26$ ), (Con and WD group  $n=11$  and HF  $n=12$ ) (Figure 2.11).



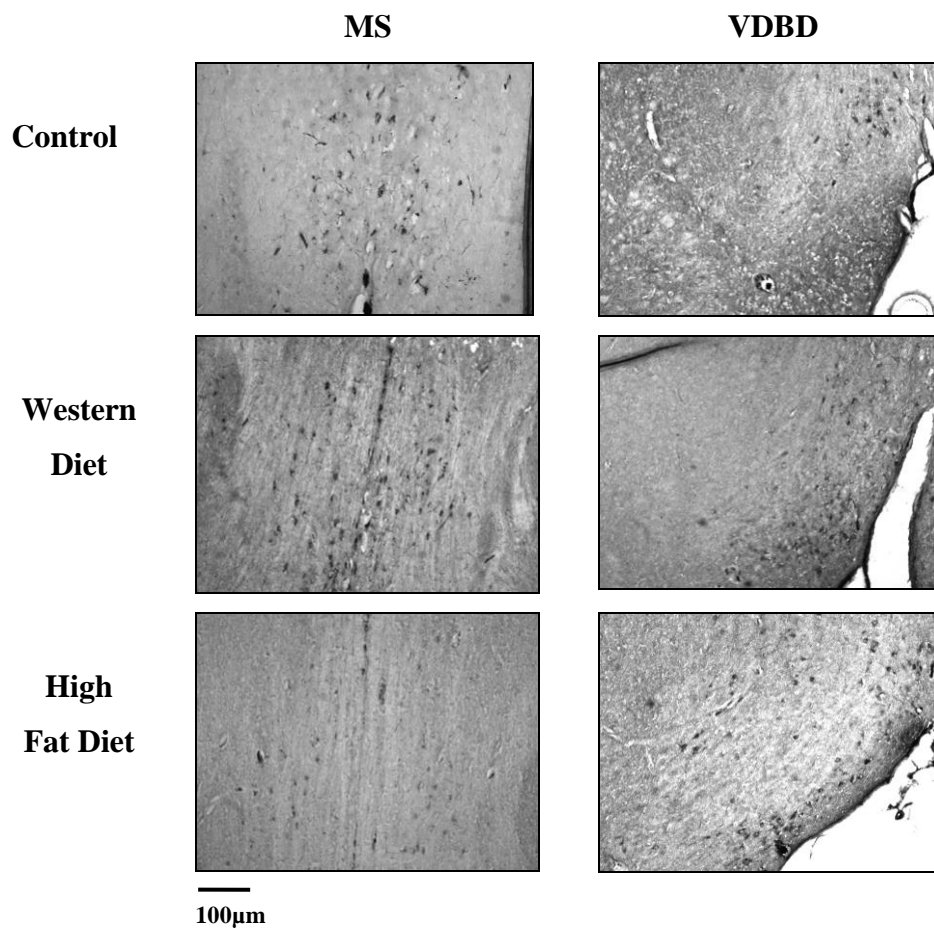
**Figure 2.10** Relative optical density of acetylcholinesterase staining in CA1, CA2&3 and dentate gyrus (DG) regions of the hippocampus, and striatum of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks;

A significant difference in acetylcholine esterase activity was observed among the groups ( $F(1,27)=3.5$ ,  $p<0.05$ ), (CA1; Con and WD  $n=12$ , HF  $n=9$ , CA2&3; Con and WD  $n=12$ , HF  $n=8$ , DG; Con and WD  $n=12$ , HF  $n=10$ , ST; Con= $12$ , WD and HF  $n=10$ ). A significant increase in acetylcholine esterase activity in the striatum of the rats was observed in the high fat diet group compared with the control group ( $p<0.05$ ). Values are expressed as mean  $\pm$  SE. \* $p<0.05$ .

A.



B.



**Figure 2.11 A. Number of Choline acetyltransferase immunoreactive neurons in medial septum (MS) and vertical limb of the diagonal band (VDBD) of rats fed control (Con), western diet (WD) or high fat (HF) diet for 12 weeks. B. Immunohistochemical staining for ChAT in VDBD and MS areas;**

No differences were observed in MS ( $F(2,33)=2.4$ ;  $p=0.11$ ), (Con and HF group  $n=12$  and WD  $n=10$ ) or VDBD area ( $F(2,33)=1.4$ ,  $p=0.26$ ), (Con and WD group  $n=11$  and HF  $n=12$ ). Values are expressed as mean + SE. B. Sample sections stained for choline acetyltransferase.



## **2.4. Discussion**

In this chapter the effects of a western and a high fat diet were investigated on memory function in adult male Long Evans rats. Consumption of a western diet (21% fat) for twelve weeks caused a significant increase in body weight, blood pressure and triglyceride level and impaired spatial, but not working memory. Similarly, a high fat diet (60% fat) impaired spatial, but not working memory; however, this effect was independent of changes in body weight or blood pressure level. Rats that received 60% fat diet for three months had significantly higher caloric intake than the control group while there was no significant difference in their body weight, blood pressure or metabolic markers. Moreover, those rats that consumed high fat diet showed an increased level of acetylcholine esterase in their striatum but not in their hippocampus.

This study is the first to report memory impairment in rats using a Y-maze paradigm (which is a one trial-one test procedure) as the result of higher percentage of fat in diet (Kosari et al., 2012). Previously, spatial memory impairments have been reported using multi-trial tasks as the result of change in the diet. For example female adult Fischer rats developed spatial memory impairment in water maze after one and two months of consuming a high fat, refined sugar diet (Molteni et al., 2002). Male Sprague Dawley rats that consumed high cholesterol diet for five months showed spatial learning deficits in 8-arm radial arm maze (Molteni et al., 2002, Ullrich et al., 2010). Impairment in spatial memory followed by a high fat and sugar diet has been observed in young (Goldbart et al., 2006) and aged (Granholt et al., 2008) rats. Young rats that fed a diet with only higher percentage of fat (similar to the high fat diet in this study) demonstrated impairment in spatial memory following the consumption of 20% lard-based diet (Greenwood and Winocur, 1990, 1996) and 58% high fat diet (Pathan et al., 2008).

In contrast to the observed spatial memory deficit, this study observed no significant impairment in object recognition paradigm followed by western or high fat diet consumption in rats. This is consistent with other studies that showed 60% fat and refined carbohydrate diets used by Flinder Resistant Line rats did not lead to deficit in object recognition task with 24 hours intertrial interval (Abildgaard et al., 2011). Interestingly, few studies suggested the quicker emergence of spatial memory deficits compared to non-spatial deficits (Broadbent et al., 2004). Object recognition was not impaired until at least 75% of the total volume of the hippocampus had been destroyed as reported by Broadbent et al in 2004 while damage to 30% to 50% of hippocampus volume was sufficient to impair spatial memory (Broadbent et al., 2004). However, working memory impairments in water maze have been observed in adult rats that consumed a high fat and sucrose diet (Hoane et al., 2011) and aged animals fed a high saturated fat diet (Granholm et al., 2008).

It is interesting to note that the impairment in spatial memory observed in this study was independent of a significant change in body weight as observed in the group that received high fat diet (60% fat). Some studies demonstrated that rats received a western diet showed increases in body weight, blood pressure, cholesterol and triglyceride levels (Dhalla et al., 2007, Liu et al., 2011). Other studies showed a significant increase in body weight followed by consumption of 60% fat diet for ten weeks in Wistar rats (Jebelovszki et al., 2008) and 70% fat diet for twelve weeks in Sprague Dawley rats (Touati et al., 2011). Long Evan rats fed a 60% high fat diet for four weeks gained significantly more body weight compared to the control group (Thaler et al., 2012). However, in this study, the 60% fat diet did not cause any change in blood pressure or metabolic markers and even the body weight of the rats slightly decreased (with an increase in the caloric intake). It is not clear why these rats did not gain more weight

while this observed lack of weight gain may be attributed to the lack of palatability of this diet and the fact that those rats did not show interest in consuming it.

The other interesting finding of this study is that the impairment observed in spatial memory was independent of a significant change in blood pressure at least in the group of rats that received high fat diet. In humans, hypertension in middle age is correlated with mild cognitive impairment (Knopman et al., 2001). Other studies demonstrated that high consumption of cholesterol in adult (mid-life) can lead to cognitive deficit in late life (Solomon et al., 2009a, Solomon et al., 2009b). The fact that in this study the blood cholesterol level of the high fat diet group was not significantly higher than control may suggest that the relationship between cholesterol level and cognitive deficit is not as strong as it was previously considered. This relationship can be influenced by many other elements such as age, cardiovascular disease and other factors (Gorelick et al., 2011).

It is still unknown how high fat diet consumption can affect memory. It has been shown that hippocampus plays an important role in spatial memory (Morris et al., 1982) and we suggest that high fat diet consumption can have degenerative effects on hippocampus. This assumption has been supported by various studies including a series of experiments that showed four weeks of feeding a high fat diet (42% fat) independent of obesity caused a decreased hippocampal neurogenesis in male rats as analysed by quantification of BrdU-immunoreactive cells (Lindqvist et al., 2006). Another research demonstrated a decrease in hippocampal long-term potentiation in high fat (45% fat) fed male mice (Porter et al., 2010, Porter et al., 2012). Level of brain derived neurotrophic factor was reduced in the hippocampus of rats that consumed high fat diet for two months (Molteni et al., 2002). Mice that consumed high fat diet for five months

showed reduced hippocampus signalling compared to the control group (Muller et al., 2008). Moreover, another study suggested that hippocampal-dependant memory deficit in rats fed high fat and high cholesterol diet occurred as the result of changes in the structure of the hippocampus of those rats due to the increase in the level of occludin in the CA3 and dentate gyrus region of the hippocampus reported by Freeman and colleagues (Freeman and Granholm, 2012). Occludin is a tight junction protein that has an essential role in formation and functioning of epithelial and endothelial barriers (Aijaz et al., 2006). The increase in the level of occludin was accompanied by a rise in the level of activated microglia in the hippocampus of rats that consumed a high fat diet (Freeman and Granholm, 2012), shows the possible influence of inflammation on hippocampus dysfunction in high fat feeding (Hwang et al., 2009). Moreover, it has been shown that consumption of a high fat diet in rats induces reactive oxygen species in the cerebral cortex (Zhang et al., 2005). This can cause cellular damage by oxidation of important cellular components including membrane lipids and proteins and finally may lead to neuronal death (Markesbery and Carney, 1999, Halliwell, 2001, See and Loeffler, 2001).

This study revealed a significant difference in acetylcholinesterase level among the groups and this difference was due to the significant increase in the level of acetylcholinesterase in the striatum of the rats that consumed a high fat diet compared to rats that received a control diet. Acetylcholinesterase is responsible for the breakdown of acetylcholine to choline and acetic acid therefore an increase in the level of this enzyme can indicate the presence of less acetylcholine in the region. Moreira and colleagues recently investigated the effect of higher level of fat and cholesterol in diet showing a higher level of acetylcholinesterase in the hippocampus is an early occurrence after consumption of a high fat/cholesterol diet (20% fat and 1.25%

cholesterol) in mice which was followed by an impairment in short-term object location memory (Moreira et al., 2014). Previously, it has been reported that scopolamine, a muscarinic cholinergic receptor antagonist can impair the object location memory in mice (Murai et al., 2007). Moreover, Assini et al. showed that an acetylcholinesterase inhibitor (Tacrine) can improve object location memory when administered before the acquisition phase of this paradigm (Assini et al., 2009). Other studies reported a decrease in the level of acetylcholine esterase in the hippocampus of rats that consumed a high fat diet for six months (Kaizer et al., 2004). A high cholesterol diet (5% cholesterol) for five months caused a decrease in the number of ChAT-positive neurons in the basal nucleus of Meynert (Ullrich et al., 2010). It has been suggested that cholesterol may modulate the activities of the cholinergic receptors by interacting with their membrane environment (Bohr, 2004). Further analysis in this study on the effect of high fat and western diet on cholinergic activity revealed no difference in the number of ChAT-positive cells in medial septum and vertical limb of the diagonal band of the rats that consumed any of the three diets. It is important to mention that cholinergic afferents from the medial septum and diagonal band densely innervates the hippocampus (Dutar et al., 1995) the main brain region involved in spatial memory regulation. This observation together with the fact that no change was also observed in the level of acetylcholinesterase in the hippocampus of rats that consumed western and high fat diet for twelve weeks, suggest that acetylcholine level in hippocampus may not play a critical role in the observed spatial memory impairment.

Therefore, this study is the first that revealed western and high fat diet can affect memory function significantly independent of body weight, blood pressure and

cholinergic changes in specific brain areas in rats. Further studies are required to investigate the mechanisms of the effect of diet on brain.

### **3. Chapter 3: Effect of western diet on memory, neuronal activation and astrocytes**

#### **3.1. Introduction**

I have demonstrated in chapter 2 that rats fed a western diet for twelve weeks show spatial memory impairment in the Y-maze; but not a working memory deficit, as assessed using the novel object recognition task (Kosari et al., 2012). However, it is still unknown at what time point during the course of the feeding, the impairment in spatial memory begins.

As has been mentioned in chapter 1, previous studies revealed that obesity can affect basal neuronal activity in specific areas of the hypothalamus (Lin and Huang, 1999). Some studies demonstrated that the induction of c-fos protein (a measure of increased neuronal activity) occurs after learning tasks. It is still unknown if consumption of a western diet can alter the neuronal activities in other brain areas, especially those that are important for memory regulation.

Moreover, there are reports that astrocyte numbers in the hypothalamus are changed in obesity (Hsuchou et al., 2009a, Berkseth et al., 2014). However, it is unknown if consumption of a high fat diet in animal models can affect astrocyte numbers in other brain areas that play a role in memory regulation.

The hippocampus, prefrontal cortex, nucleus accumbens and paraventricular nucleus of thalamus are regions of the brain that are involved in memory regulation. Studies showed that, CA1 (cornu ammonis 1), CA2&3 (cornu ammonis 2&3) and dentate gyrus (DG) of the hippocampus are specifically important for spatial location

information and lesion of the hippocampus causes impairment in spatial navigation tasks in rodents (Morris et al., 1982, Long and Kesner, 1996, Whishaw et al., 1997, Long and Kesner, 1998, Moser and Moser, 1998). These patterns of connectivity in prefrontal cortex, enables this region to integrate a wide range of information required for complicated behaviour and influence many brain processes. Prefrontal cortex is particularly well known for its involvement in executive function such as decision making and specifically in working memory (Miller, 2000, Miller and Cohen, 2001) and its connection with the hippocampus (Mendez et al., 2008, Kesner and Churchwell, 2011). Other areas of interest are the anterior thalamic nuclei and nucleus accumbens that are important in memory due to the pathways existing between the hippocampal formation, prefrontal cortex, amygdala and those brain regions (Yasoshima et al., 2007, Aggleton et al., 2010).

The present chapter investigates the changes that occur in the basal neuronal activity in different brain regions important for memory function following the consumption of a western and control diet. Furthermore, it determines whether consumption of a western diet produces changes in astrocyte number by detecting glial fibrillary acidic protein (GFAP) as an astrocyte marker, in brain regions involved in memory regulation and feeding in rats.



## **3.2. Materials and methods**

### **3.2.1. Animals and chow**

Male Long Evans rats (Monash University, Australia) were housed in groups of two under a light/dark cycle (12 hour/12 hour), in a temperature controlled room (22°C) with food and water *ad libitum*. Animals were randomly divided into two groups (n=12) and assigned to either a control (Con; Control for SF00-219, SF04-057 rodent diet, 6% Fat Semi-Pure Rodent Diet including 0.43% total saturated fatty acids; Specialty Feeds, Perth, Australia) or western diet (WD; SF00-219, 21% total fat including 1.80% total saturated fats and 0.15% cholesterol; Specialty Feeds). Animals received the diets for 15 weeks and behavioural testing was conducted every 4 weeks. Food intake and body weight were measured twice-weekly. Caloric intake was calculated from food intake. All experimental protocols were performed in accordance with the Prevention of Cruelty to Animals Act 1986 (Australia), the National Health and Medical Research Council (NHMRC) code EA28 and approved by the RMIT University Animals Ethics Committee.

### **3.2.2. Behavioural testing**

#### *Novel object recognition test*

Methods were as described in chapter 2.2.3.1. The tests were repeated at the end of each month of feeding. The novel objects used in this paradigm were different each month.

### *Y-maze*

Methods were as described in Chapter 2.2.3.2. The tests were repeated at the end of each month of feeding.

### **3.2.3. Collection of blood and brain**

Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.). Blood was collected, centrifuged at 3000 RPM for 20 minutes at 6°C and the serum transferred to -20°C. Rats were perfused with freshly prepared ice cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS). After perfusion brains were removed and placed in 4% PFA for 3 hours at room temperature then transferred to 30% sucrose solution. Coronal sections (30µm) thickness were cut on a cryostat (Leica CM 1900 Microsystems Nussloch GmbH, Germany) and stored in cryoprotectant (37.5% sucrose, 1.25% polyvinyl pyrrolidine, 37.5% ethylene glycol in 0.1M phosphate buffered saline (PBS)) in 24 well plates until required for analysis. Serial coronal sections (30 µm thick) of the brain were cut using a cryostat (Leica CM 1900 Microsystems Nussloch GmbH, Germany). The sections were collected and kept in cryoprotectant (37.5% sucrose, 1.25% polyvinyl pyrrolidine, 37.5% ethylene glycol in 0.1M phosphate buffered saline) in 24 well plates to detect c-fos and GFAP using standard immunohistochemical procedures.

### **3.2.4. C-fos immunohistochemistry**

In order to identify activated neurons, immunohistochemistry for the c-fos protein was performed using free floating method. Staining started by washing the sections in PBS 5 times for 30 minutes duration in total. Endogenous peroxidase activity was blocked by incubating with 0.5% H<sub>2</sub>O<sub>2</sub> for 30 minutes followed by three washes in PBS for five

minutes duration each. Sections then were incubated for 60 minutes in 10% normal goat serum (NGS) proceeded by incubation in 0.5% Triton X-100 (10 minutes) to facilitate the penetration of antibody. This step followed by three washes in PBS (5 minutes each) and incubation in anti-fos primary antibody (rabbit polyclonal IgG, c-fos (K-25): sc-253, Santa Cruz Biotechnology, CA, USA; dilution: 1:400) for 24 hours at 4 °C. Then sections were washed again three times in PBS for five minutes each and were incubated for 1 hour with biotinylated secondary antibody (1:600, anti-rabbit raised in goat, B8895, Sigma Aldrich, Australia) diluted in NGS (2%) followed by another three washes in PBS and a two hours incubation in ABC kits (Vector laboratories, VECTASTAIN, ABC kit) in room temperature. The ABC solution was prepared at least 30 minutes before the incubation. Then sections were washed twice (for 10 minutes duration in total) and incubated in 3,3' Diaminobenzidine (DAB) kit (Vector laboratories, DAB Peroxidase kit e Substrate. Cat. No. SK-4100), until the colour of the sections changed and the staining was visible. Before the sections become too dark the reaction was stopped by putting the sections in dH<sub>2</sub>O. Sections were mounted on the gelatine coated slides and passed through an increasing gradient of alcohol followed by xylene then sections were cover-slipped using Depex mounting medium.

Six images were captured from each region of interest. c-fos immunoreactive neuron counts were performed using Image J program. The averages were calculated and analysis of variance (ANOVA) tests were performed using procedures for general linear models with operations for repeated measures and simple effects.

### **3.2.5. GFAP immunohistochemistry**

In order to identify astrocytes, immunohistochemistry for the GFAP protein was performed using free floating method. Staining began by washing the sections in PBS for five minutes. Endogenous peroxidase activity was blocked by incubating with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 minutes followed by three washes in PBS for five minutes duration each. Sections then were incubated for 60 minutes in 10% normal goat serum (NGS) proceeded by three washes in PBS (5 minutes each) followed by incubation in anti-GFAP primary antibody (rabbit anti-cow GFAP, Dako, Denmark, 1:1000 dilution (diluted in 2% NGS and 0.2% Triton X-100 in PBS)) for 24 hours at 4°C. Then sections were washed again three times in PBS for five minutes each and were incubated for 45 minutes with biotinylated secondary antibody (goat biotinylated anti-rabbit IgG (Vector laboratories, 1:100 dilution) diluted in 2% NGS and 0.2% Triton X-100 in PBS followed by another three washes in PBS and a two hours incubation in ABC kits (Vector laboratories, VECTASTAIN, ABC kit) in room temperature. The ABC solution was prepared at least 30 minutes before the incubation. Then sections were washed twice (for 10 minutes duration in total) and incubated in DAB kit (Vector laboratories, DAB Peroxidase kit e Substrate. Cat. No. SK-4100) until the colour of the sections changed and the staining was visible. Before the sections become too dark, the reaction was stopped by putting the sections in dH<sub>2</sub>O. Sections were mounted on the gelatine coated slides and passed through an increasing gradient of alcohol followed by xylene then they were cover-slipped using Depex mounting medium.

Six images were captured from each region of interest. The GFAP positive cells were quantified using Image J program.

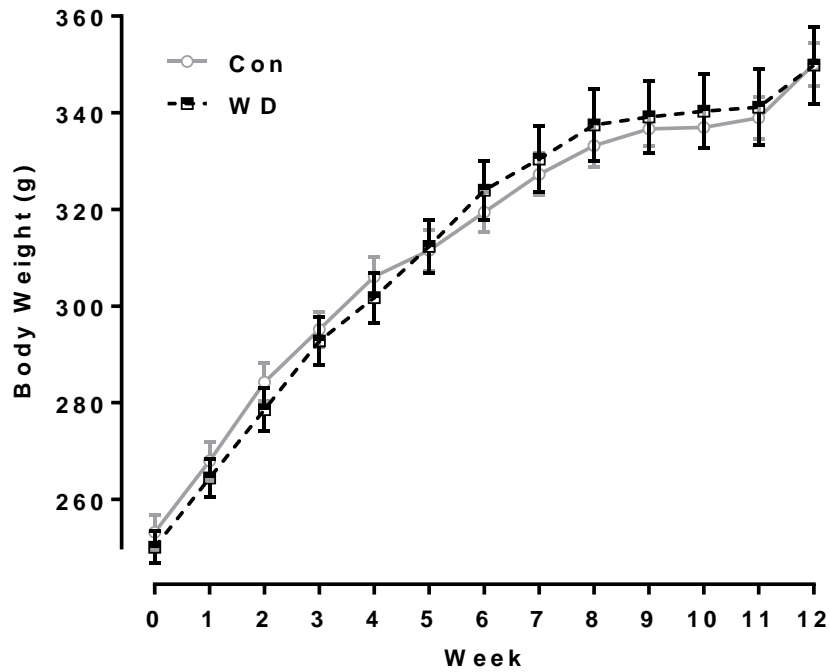
### **3.2.6. Statistical analysis**

All data were analysed using SPSS for Windows Version 18.0. Body weight, food intake, and calorie intake were analysed using two-way ANOVA with group and week as factors; when applicable, simple effects for each group were analysed. Fasting blood glucose and epididymal fat pad weight values were assessed using Student's t-test. Exploration times for the novel object recognition test were analysed using two-way ANOVA with group and object as factors; while discrimination ratios were evaluated using one-way ANOVA. To analyse the Y-maze result, time and entries of the novel arm were determined by one-way ANOVA. GFAP-positive astrocytes and c-fos-positive cells were analysed using two-way ANOVA with group and region as factors; simple effects for each group were determined when applicable.

## **3.3. Results**

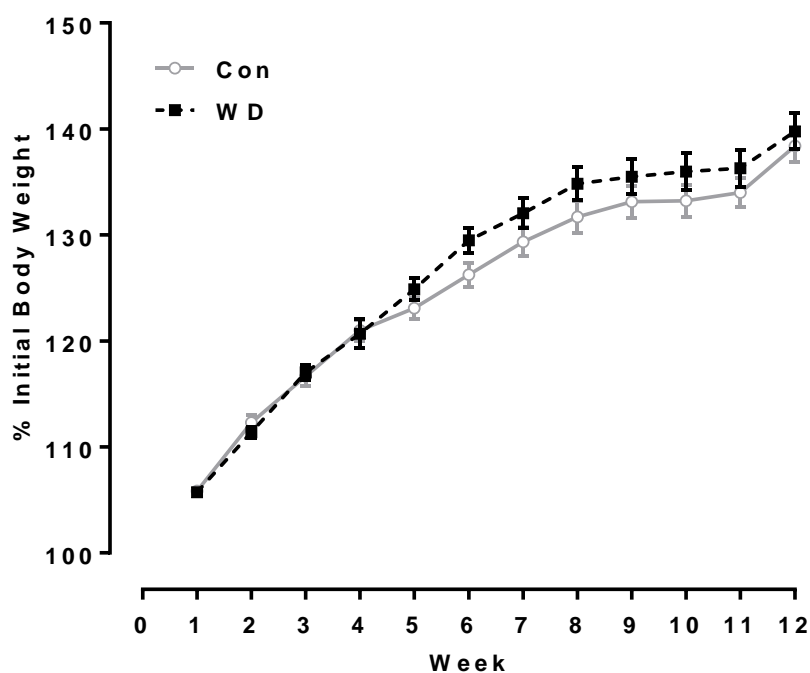
### **3.3.1. Effect of diet on body weight, food and calorie intake**

Body weights at the beginning of the dietary feeding regimen were not significantly different among the two groups. Analysis of the body weight over the 12 experimental weeks demonstrated no significant difference between the two groups (Group:  $F(1,22) = 0.000$ ,  $p=0.99$ , Week:  $F(1,22) = 725.5$ ,  $p<0.001$ , Week x Group:  $F(1,22) = 1.2$ ,  $p=0.28$ ). Moreover, no significant difference was observed in the percentage of initial body weight over the 12 weeks (Group:  $F(1,22) = 1.0$ ,  $p=0.335$ , Week:  $F(1,22) = 693.2$ ,  $p<0.001$ , Week x Group:  $F(1,22) = 1.7$ ,  $p=0.21$ ). However, the caloric intake of the two groups were significantly different (Group:  $F(1,22) = 117.92$ ,  $p<0.001$ , Week:  $F(1,22) = 1143.58$ ,  $p<0.001$ , Week x Group:  $F(1,22) = 39.25$ ,  $p<0.001$ ).



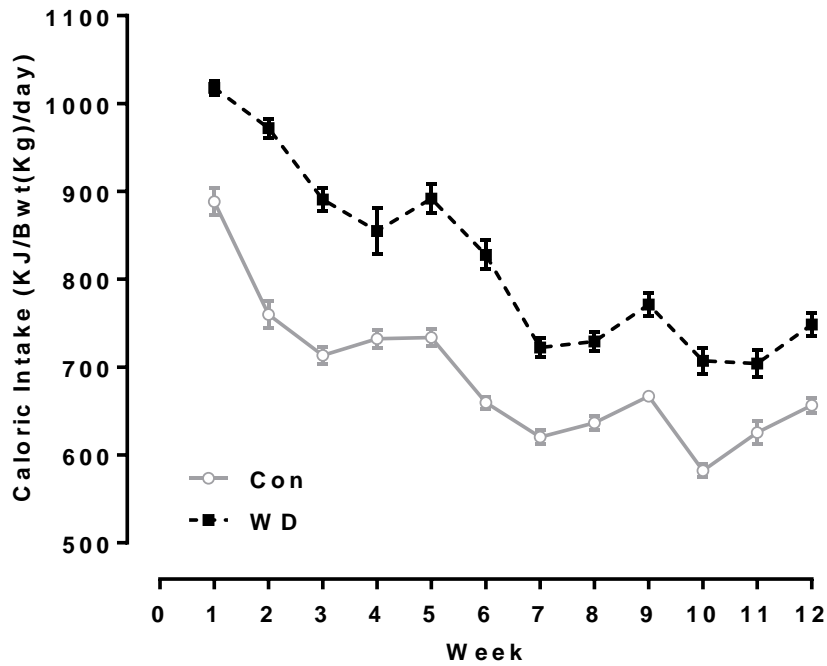
**Figure 3.1 Body weight (g) of rats fed control (Con) or western diet (WD) for 12 weeks;**

Analysis of the body weight over the 12 experimental weeks demonstrated no significant difference between the two groups (Group:  $F(1,22) = 0.000$ ,  $p=0.99$ , Week:  $F(1,22) = 725.5$ ,  $p<0.001$ , Week x Group:  $F(1,22) = 1.2$ ,  $p=0.28$ ).  $n=12$ . Values are expressed as mean + SE.



**Figure 3.2 Percentage of the initial body weight of rats fed control (Con) or western diet (WD) for 12 weeks;**

No significant difference was observed in the percentage of initial body weight over the 12 weeks between the groups (Group:  $F(1,22) = 1.0$ ,  $p=0.335$ , Week:  $F(1,22) = 693.2$ ,  $p<0.001$ , Week x Group:  $F(1,22) = 1.7$ ,  $p=0.21$ ).  $n=12$ . Values are expressed as mean + SE.



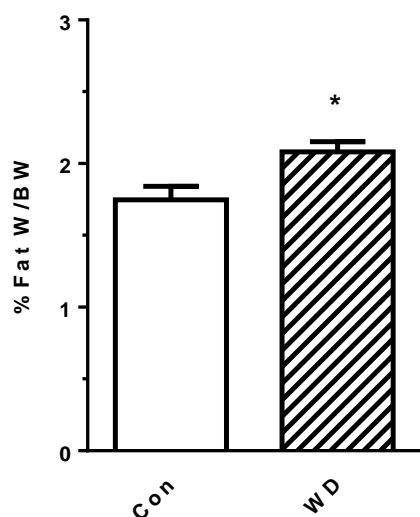
**Figure 3.3 Caloric intake (KJ/Body weight (Kg)/day) of rats fed control (Con) or western diet (WD) diet for 12 weeks;**

The caloric intake of the two groups were significantly different (Group:  $F(1,22) = 117.92$ ,  $p < 0.001$ , Week:  $F(1,22) = 1143.58$ ,  $p < 0.001$ , Week x Group:  $F(1,22) = 39.25$ ,  $p < 0.001$ ).  $n=12$ . Values are expressed as mean  $\pm$  SE.

### 3.3.2. Effect of diet on blood glucose and epididymal fat pad

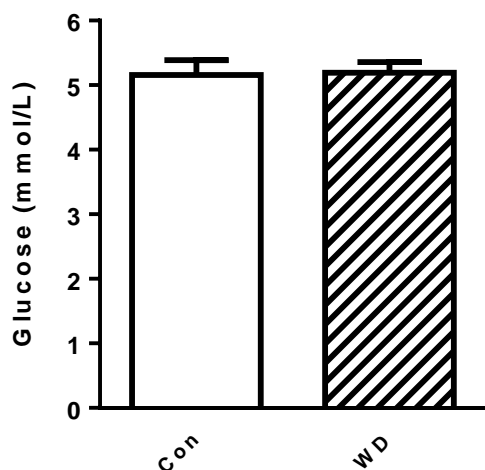
Analysis of epididymal fat pad revealed a significant increase in the percentage of fat weight/body weight of rats that consumed a western diet compared to the control group ( $p=0.01$ ). No significant difference was observed in the fasting blood glucose level of the two groups ( $p=0.91$ ).





**Figure 3.4 Epididymal fat pad analysis. Percentage of Fat weight/Body weight of rats fed control (Con) or western diet (WD) for 12 weeks;**

A significant increase was observed in the percentage of fat weight/body weight of rats that consumed a western diet compared to the control group.  $n=12$ . Values are expressed as mean  $\pm$  SE. \* $p=0.01$ .



**Figure 3.5 Fasting serum glucose (mmol/L) level of rats fed control (Con) or western diet (WD) for 12 weeks;**

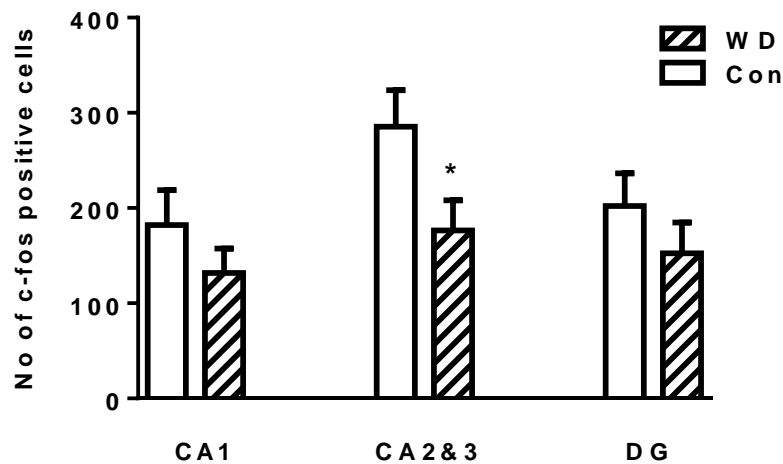
No significant difference was observed in the fasting blood glucose level of the two groups ( $p=0.91$ ).  $n=12$ . Values are expressed as mean  $\pm$  SE.

### 3.3.3. Effect of diet on basal neuronal activity

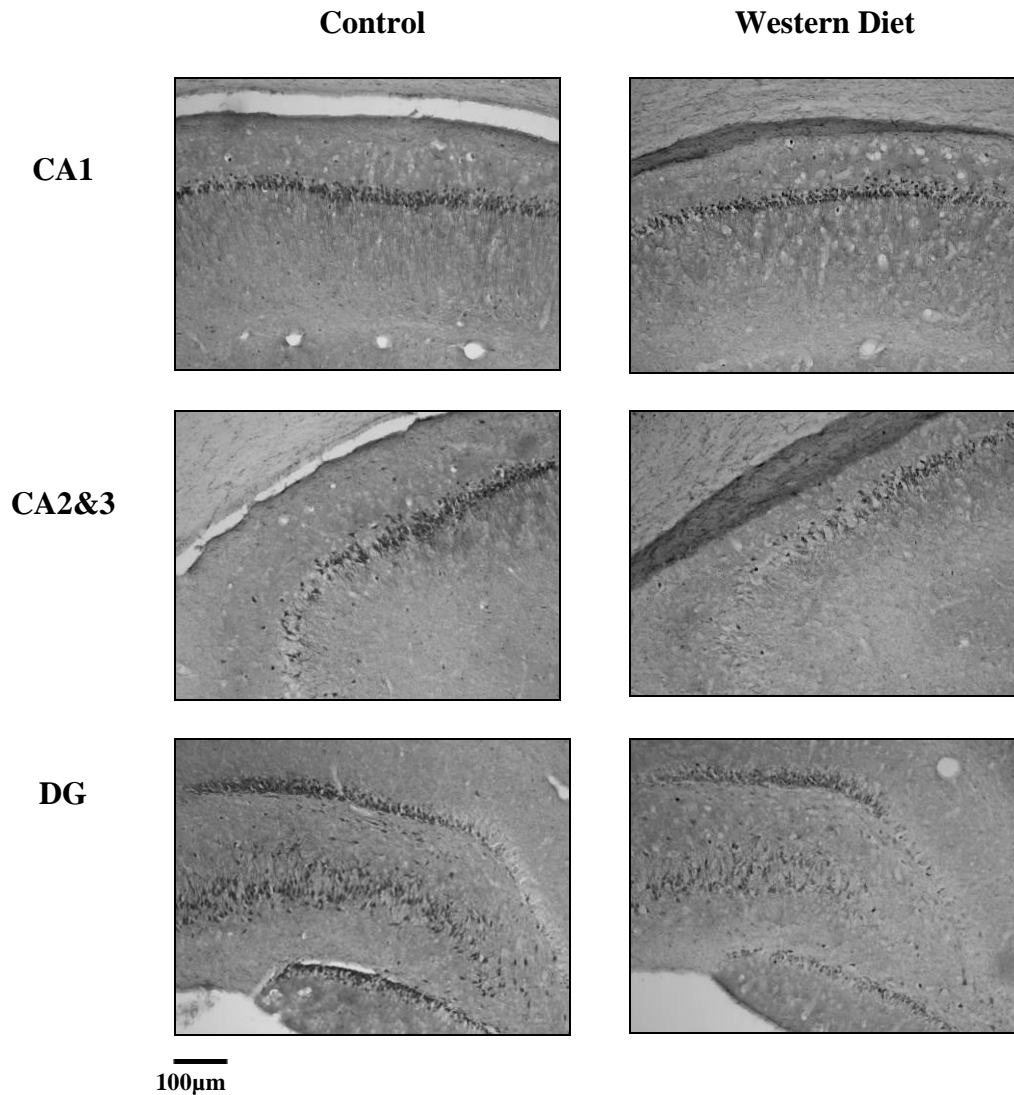
#### 3.3.3.1. Effect of diet on basal neuronal activity in the hippocampus

Consumption of a western diet for twelve weeks significantly decreased basal c-fos immunoreactivity in CA2&3 regions of hippocampus ( $F(1, 20) = 4.90, p=0.04$ ) and not in the whole region (Group:  $F(1, 21) = 0.76, p=0.39$ , Region:  $F(1, 21) = 0.05, p=0.83$ , Group x Region:  $F(1, 21) = 1.68, p=0.21$ ). Moreover, western diet did not have any significant effect on c-fos immunoreactivity in CA1 ( $F(1, 20) = 1.33, p=0.26$ ) or in dentate gyrus ( $F(1, 20) = 1.08, p=0.31$ ).

A.



B.



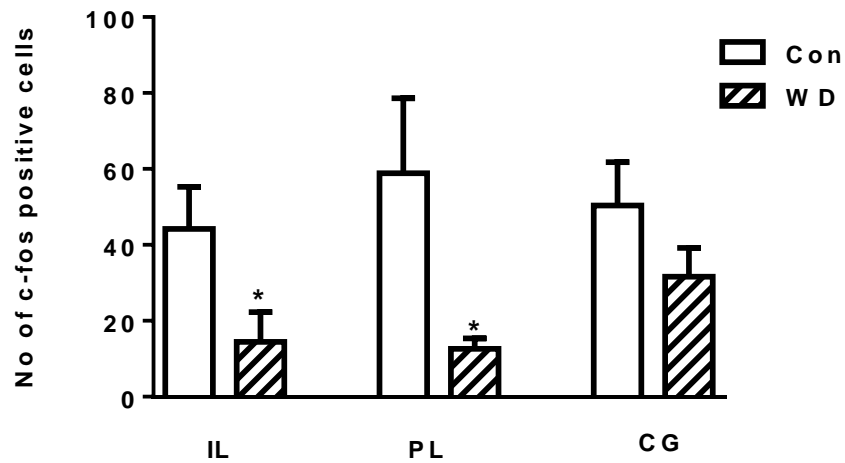
**Figure 3.6 A. Number of c-fos-positive cells in CA1, CA2&3 and dentate gyrus (DG) regions of the hippocampus of rats fed control (Con) or western diet (WD) for 12 weeks; B. Immunohistochemical staining for c-fos in the hippocampus subregions;**

Consumption of a western diet for twelve weeks significantly decreased basal c-fos immunoreactivity in CA2&3 regions of hippocampus ( $F(1, 20) = 4.90, p=0.04$ ) while it did not change the c-fos counts in the other areas or the hippocampus as a whole.  $n=12$ . \*  $p= 0.04$ . Values are expressed as mean + SE.

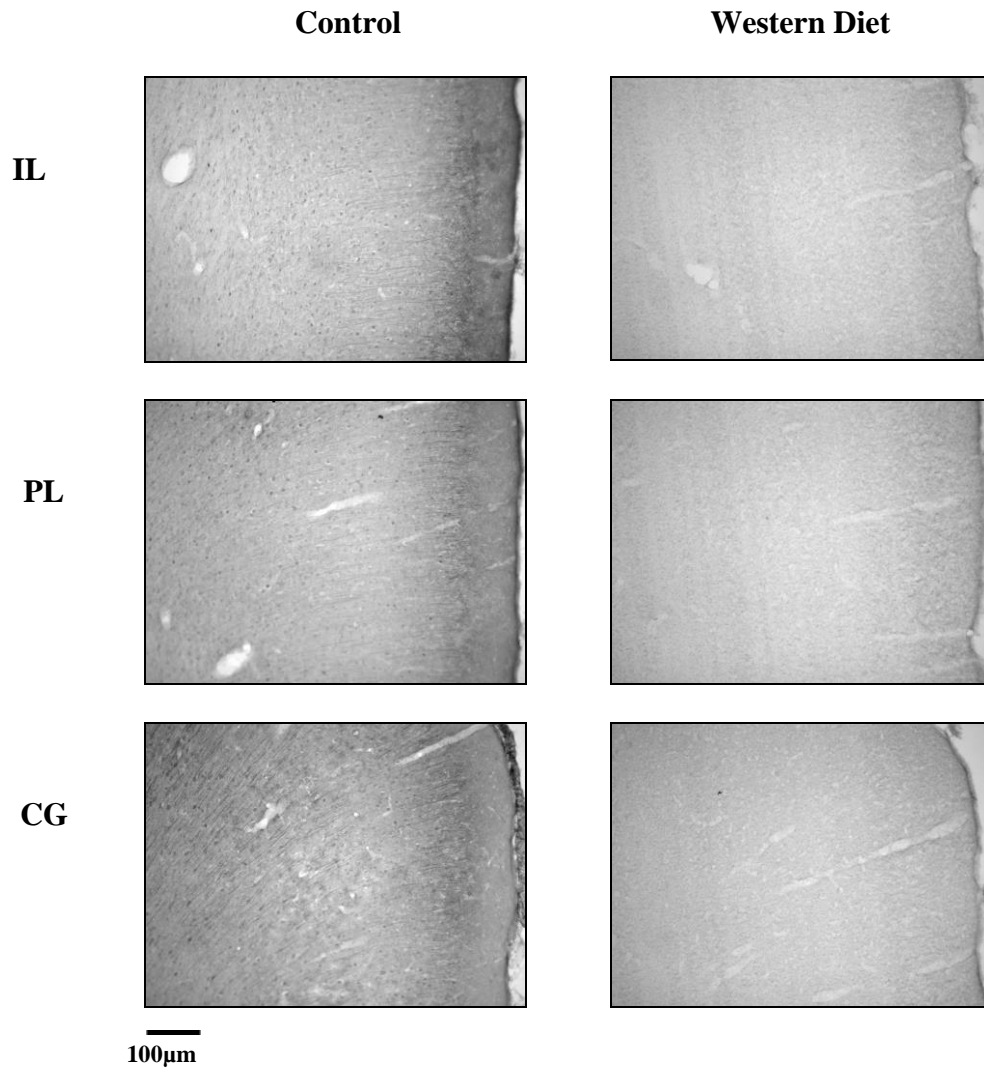
### 3.3.3.2. Effect of diet on basal neuronal activity in the prefrontal cortex

There was a significant decrease in the number of c-fos-positive cells in the infralimbic ( $F(1, 22) = 4.95, p=0.04$ ) and prelimbic region ( $F(1, 23) = 5.42, p=0.03$ ). No significant difference was observed in the number of c-fos-positive cells in the anterior cingulate of the prefrontal cortex ( $F(1, 23) = 1.89, p=0.18$ ). There was a significant group effect on the prefrontal cortex as a whole: (Group:  $F(1, 21) = 7.02, p=0.015$ , Region:  $F(1, 21) = 2.67, p=0.12$ , Group x Region:  $F(1, 21) = 0.29, p=0.60$ ).

A.



B.



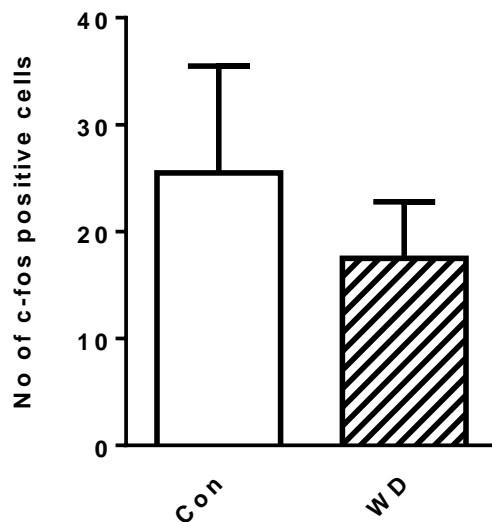
**Figure 3.7 A. Number of c-fos-positive cells in infralimbic (IL), prelimbic (PL) and anterior cingulate (CG) of the prefrontal cortex of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for c-fos in the prefrontal cortex subregions;**

A significant decrease was observed in the number of c-fos-positive cells in the infralimbic ( $F(1, 22) = 4.95, p=0.04$ ), prelimbic region ( $F(1, 23) = 5.42, p=0.03$ ) and a significant group effect on the prefrontal cortex as a whole: (Group:  $F(1, 21) = 7.02, p=0.015$ , Region:  $F(1, 21) = 2.67, p=0.12$ , Group x Region:  $F(1, 21) = 0.29, p=0.60$ ).  $n=12$ . \*  $p<0.05$ . Values are expressed as mean  $\pm$  SE.

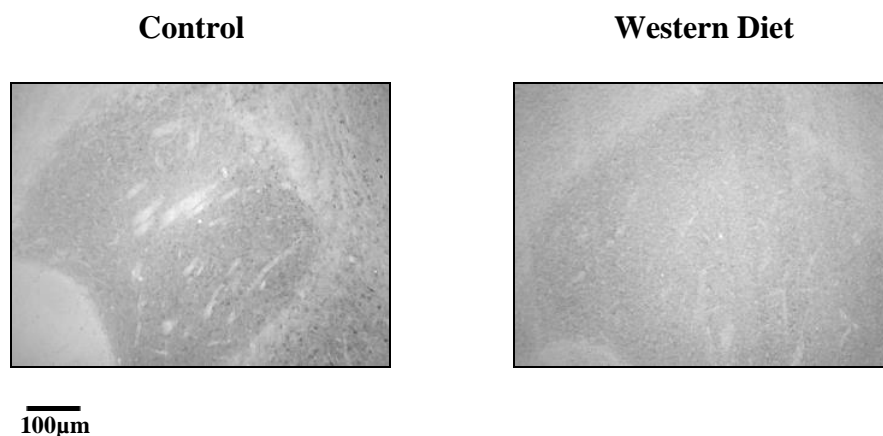
### 3.3.3.3. Effect of diet on basal neuronal activity in the nucleus accumbens

No significant difference was observed in the level of c-fos-positive cells in the nucleus accumbens of rats that consumed control or western diets ( $F(1, 23) = 0.50, p=0.49$ ).

A.



B.



**Figure 3.8 A. Number of c-fos-positive cells in the nucleus accumbens of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for c-fos in the nucleus accumbens;**

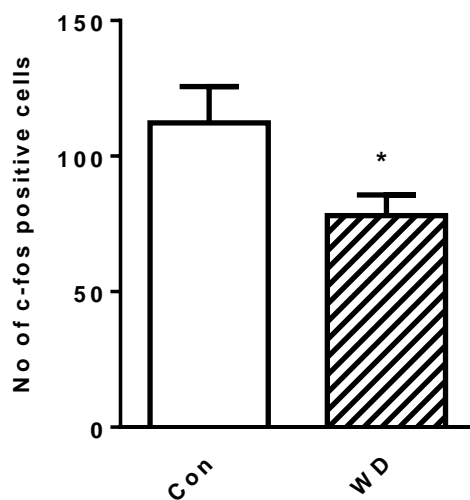
No significant difference was observed in the level of c-fos-positive cells in the nucleus accumbens of rats that consumed control or western diets.  $n=12, p=0.49$ .

Values are expressed as mean + SE.

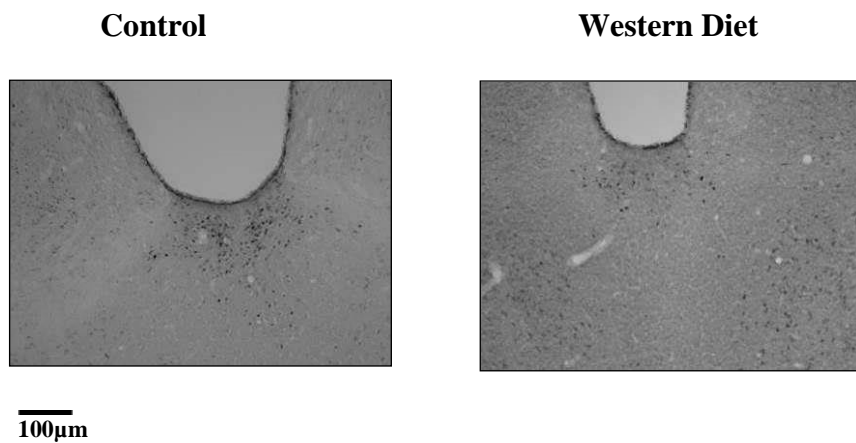
### 3.3.3.4. Effect of diet on basal neuronal activity in the paraventricular nucleus of thalamus

A significant decrease was observed in c-fos immunoreactivity in the paraventricular nucleus of thalamus of rats that consumed a western diet compared to control ( $F(1, 23) = 4.88, p=0.04$ ).

A.



B.



**Figure 3.9 A. Number of c-fos-positive cells in the paraventricular nucleus of thalamus of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for c-fos in the paraventricular nucleus of thalamus;**

A significant decrease was observed in c-fos immunoreactivity in paraventricular

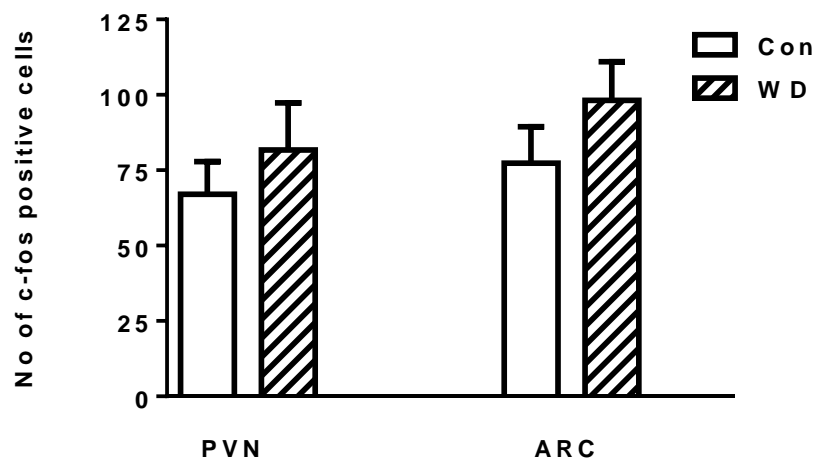
nucleus of thalamus of rats that consumed a western diet compared to control. n=12.

\*  $p=0.04$ . Values are expressed as mean  $\pm$  SE.

### 3.3.3.5. Effect of diet on basal neuronal activity in the paraventricular and arcuate nucleus of the hypothalamus

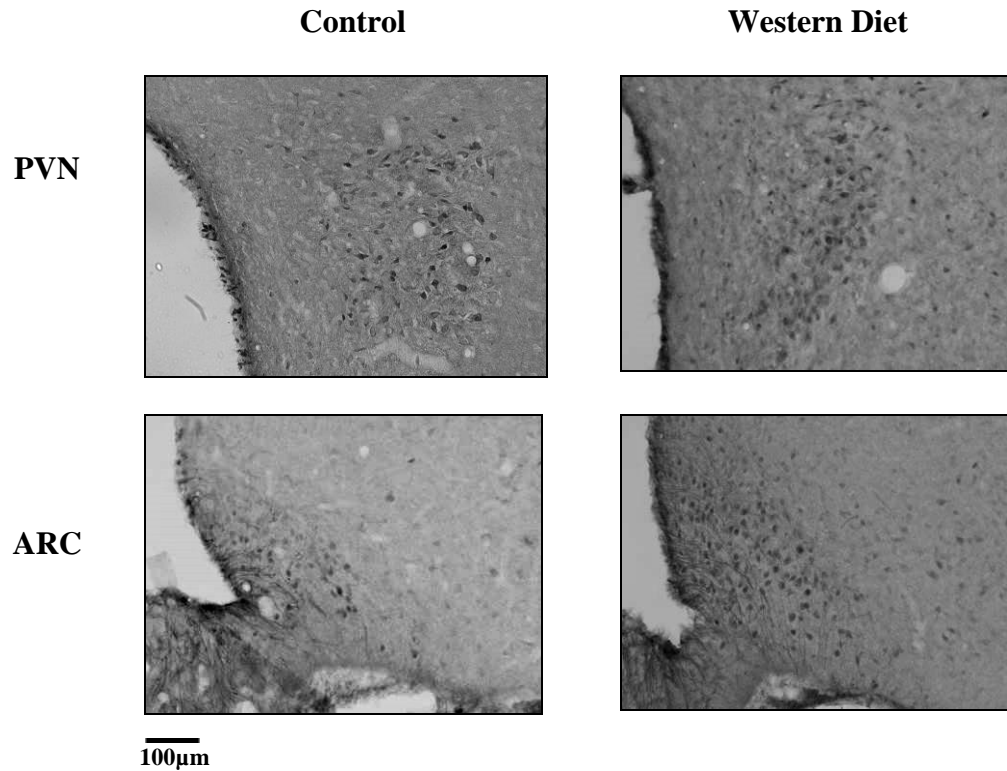
Analysis of the number of c-fos-positive cells in the hypothalamus in both paraventricular nucleus and arcuate nucleus revealed no significant difference between the western diet and control groups (Group:  $F(1, 19) = 1.19$ ,  $p=0.29$ , Region:  $F(1, 19) = 1.94$ ,  $p=0.18$ , Group  $\times$  Region: ( $F(1, 19) = 0.004$ ,  $p=0.95$ ). Analysis of the regions independently revealed no difference in the number of c-fos-positive cells in the arcuate nucleus ( $F(1, 21) = 1.36$ ,  $p=0.26$ ) as well as paraventricular nucleus ( $F(1, 22) = 0.605$ ,  $p=0.445$ ) of the hypothalamus between the groups.

A.





B.



**Figure 3.10 A. Number of c-fos-positive cells in the paraventricular nucleus (PVN) and arcuate nucleus (ARC) of the hypothalamus of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for c-fos in the hypothalamus subregions;**

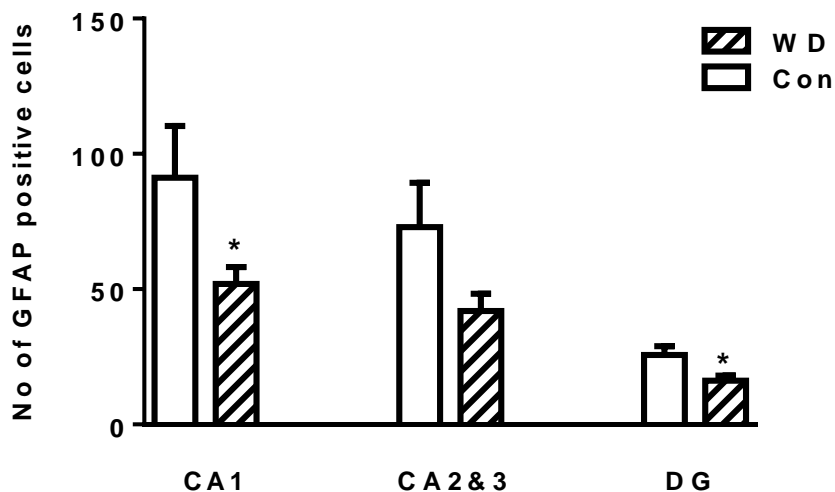
Analysis of the number of c-fos-positive cells in the hypothalamus in both paraventricular nucleus and arcuate nucleus together and independently revealed no significant difference between the western diet and control groups. n=12. Values are expressed as mean + SE.

### 3.3.4. Effect of diet on astrocytes

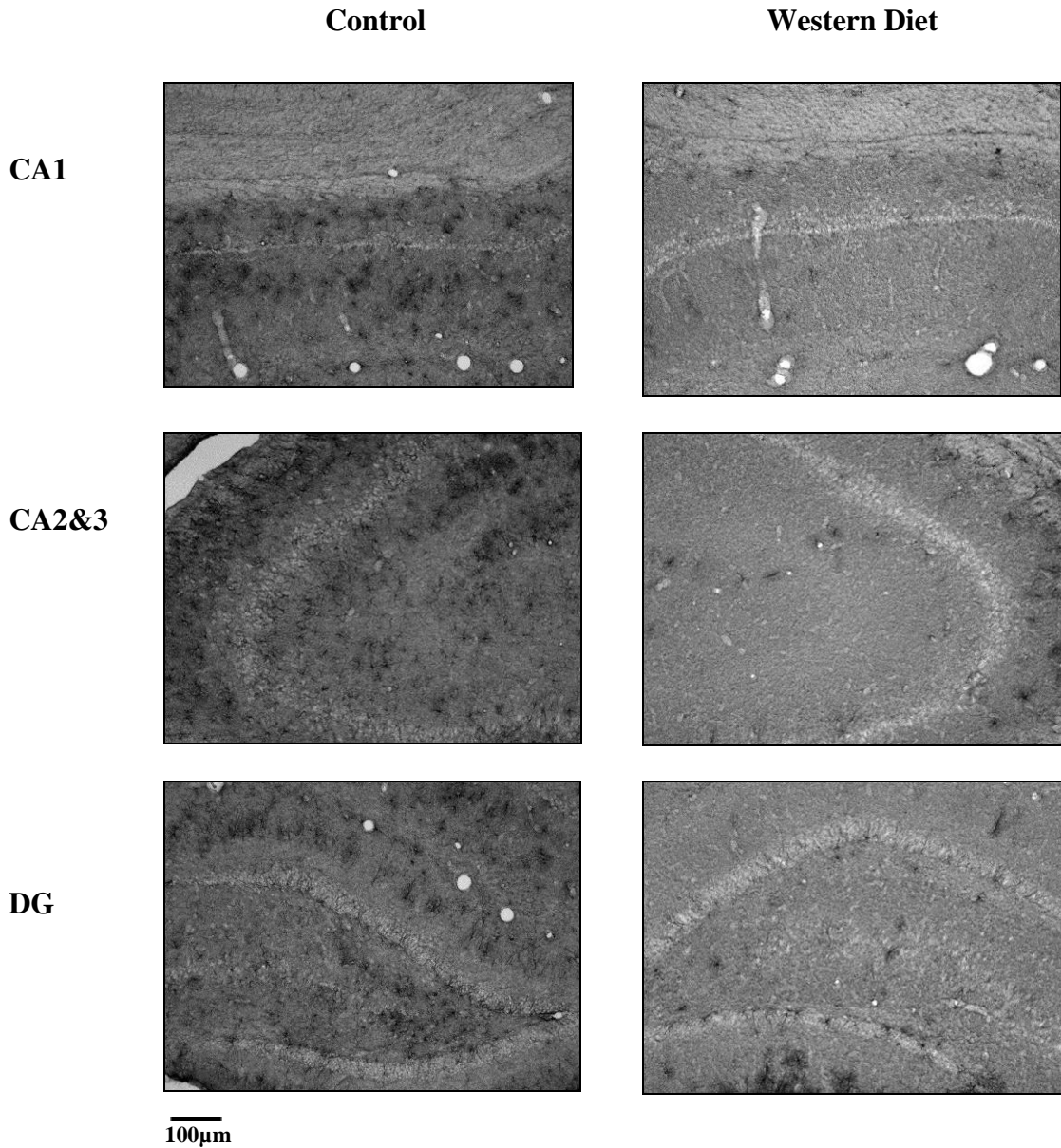
#### 3.3.4.1. Effect of diet on astrocytes in the hippocampus

Consumption of a western diet for twelve weeks significantly decreased number of GFAP-positive astrocytes in CA1 ( $F(1,21)=4.39$ ,  $p=0.049$ ) and dentate gyrus ( $F(1,21)=7.09$ ,  $p=0.015$ ) regions of hippocampus and not in CA2&3 ( $F(1,21)=3.56$ ,  $p=0.07$ ) independently. There was no significant group x region changes observed (Group:  $F(1, 20) = 4.896$ ,  $p=0.039$ , Region:  $F(1, 20) = 33.21$ ,  $p<0.001$  Group x Region:  $F(1, 20) = 2.86$ ,  $p=0.11$ ).

A.



B.



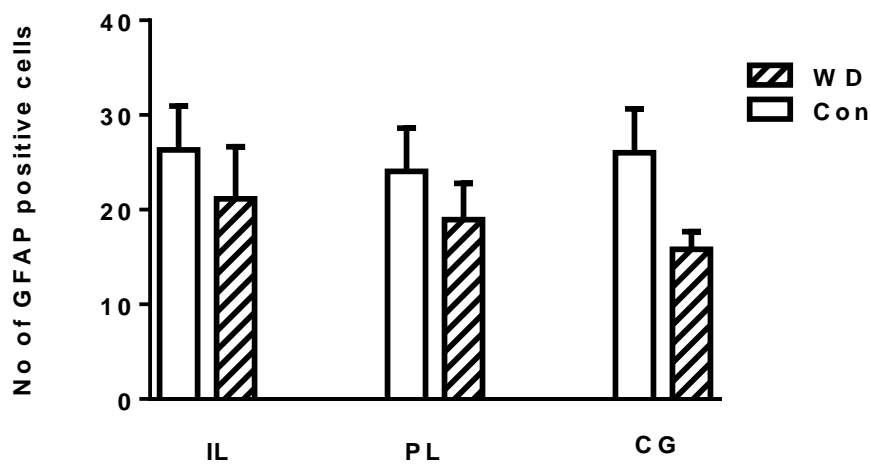
**Figure 3.11 A. Number of GFAP positive cells in the CA1, CA2&3 and dentate gyrus (DG) regions of the hippocampus of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for GFAP in the hippocampus subregions;**

Consumption of a western diet for twelve weeks significantly decreased basal astrocytes in CA1 ( $F(1,21)=4.39$ ,  $p=0.049$ ) and dentate gyrus ( $F(1,21)=7.09$ ,  $p=0.015$ ) regions of hippocampus and not in CA2&3 ( $F(1,21)=3.56$ ,  $p=0.07$ ) independently.  $n=12$ . \* $p<0.05$ . Values are expressed as mean + SE.

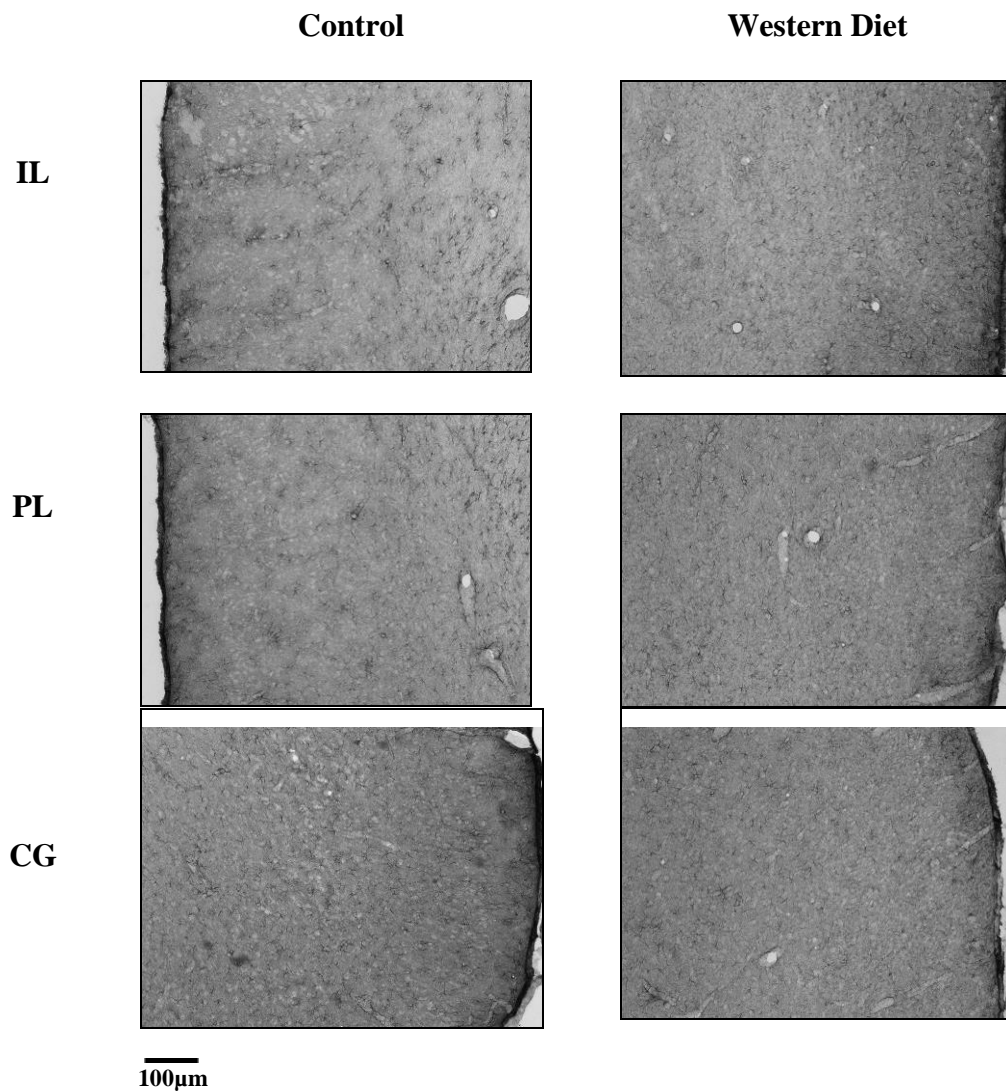
### 3.3.4.2. Effect of diet on astrocytes in the prefrontal cortex

Consumption of a western diet for twelve weeks did not cause a significant change in the number of astrocytes in the prefrontal cortex (Group:  $F(1, 22) = 1.54$ ,  $p=0.23$ , Region:  $F(1, 22) = 0.965$ ,  $p=0.34$ , Group x Region:  $F(1, 22) = 0.78$ ,  $p=0.39$ ). The results on each of those subregions revealed no significant difference in infralimbic ( $F(1,23)=0.52$ ,  $p=0.48$ ), prelimbic ( $F(1,23)=0.73$ ,  $p=0.40$ ) and anterior cingulate ( $F(1,23)=4.165$ ,  $p=0.053$ ).

A..



B.



**Figure 3.12 A. Number of GFAP positive cells in infralimbic, prelimbic and anterior cingulate of the prefrontal cortex of rats fed control (Con) or western diet (WD) for 12 weeks. B. Immunohistochemical staining for GFAP in the prefrontal cortex subregions ;**

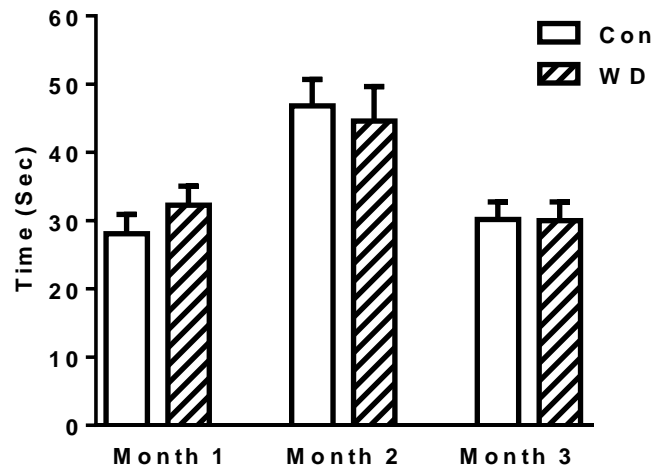
Consumption of a western diet for twelve weeks did not cause a significant change in the number of astrocytes in the prefrontal cortex as a whole or in any of its subregions independently. n=12. Values are expressed as mean + SE.

### **3.3.5. Effect of diet on novel object recognition paradigm**

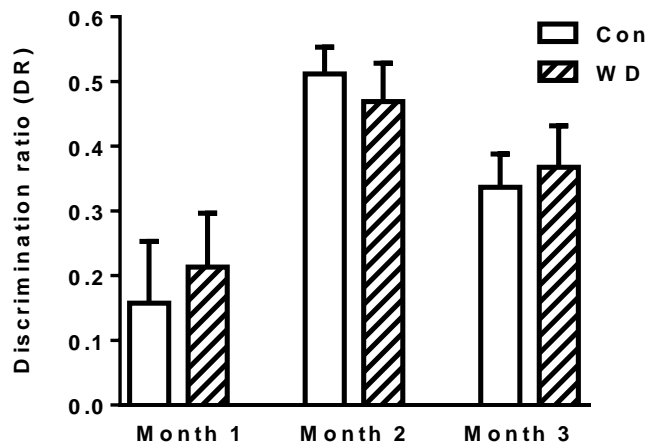
No significant difference was observed between the control and western diet groups in the time rats spent exploring the novel object in month 1: ( $F(1,23)=1.09$ ,  $p=0.31$ ), month 2: ( $F(1,23)=0.125$ ,  $p=0.73$ ), month 3: ( $F(1,23)=0.002$ ,  $p=0.965$ ) independently or overall during the experiment (Group:  $F(1, 22) = 0.035$ ,  $p=0.85$ , Month:  $F(1, 22) = 0.001$ ,  $p=0.98$ , Group x Month:  $F(1, 22) = 0.54$ ,  $p=0.47$ ).

No significant difference was observed between the control and western diet groups in the discrimination ratio in month 1: ( $F(1,23)=0.195$ ,  $p=0.66$ ), month 2: ( $F(1,23)=0.34$ ,  $p=0.56$ ), month 3: ( $F(1,23)=0.14$ ,  $p=0.71$ ) independently or overall during the 3 months of the experiment (Group:  $F(1, 22) = 0.14$ ,  $p=0.71$ , Month:  $F(1, 22) = 3.63$ ,  $p=0.07$ , Group x Month:  $F(1, 22) = 0.02$ ,  $p=0.89$ ).

A.



B.



**Figure 3.13 Novel objection recognition result of rats fed control (Con) or western diet (WD) for 12 weeks;**

A. No significant difference was observed between the control and western diet groups in the time rats spent exploring the novel object (seconds), each month independently or overall during the experiment in the trial phase.  $n=12$ . Values are expressed as mean  $\pm$  SE. B. No significant difference was observed between the control and western diet groups in the discrimination ratio, each month independently or overall during the 3 months of the experiment.  $n=12$ . Values are expressed as mean  $\pm$  SE.

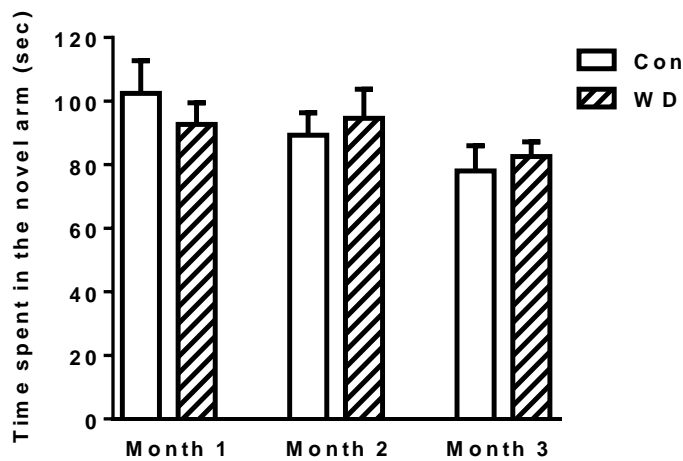
### **3.3.6. Effect of diet on Y-maze paradigm performance**

No significant difference was observed between the control and western diet groups in the time the animals spent in the novel arm in month 1: ( $F(1,23)=0.62, p=0.44$ ), month 2: ( $F(1,23)=0.21, p=0.65$ ), month 3: ( $F(1,23)=0.25, p=0.62$ ) independently or overall during the experiment (Group:  $F(1, 22) < 0.001, p=0.995$ , Month:  $F(1, 22) = 7.085, p=0.01$ , Group x Month:  $F(1, 22) = 1.22, p=0.28$ ).

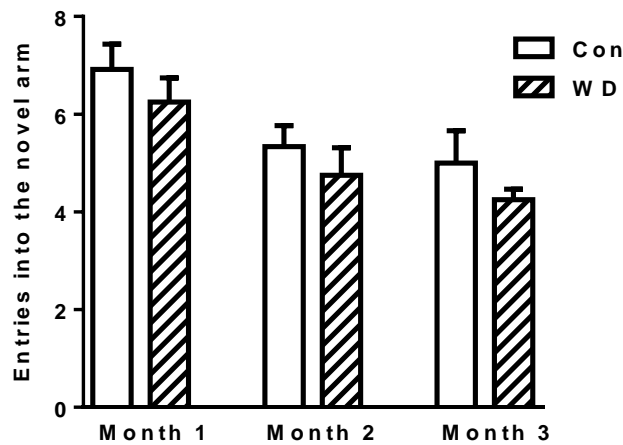
No significant difference was observed between the control and western diet groups in the number of entries into the novel arm in month 1: ( $F(1,23)=0.87, p=0.36$ ), month 2: ( $F(1,23)=0.67, p=0.42$ ), month 3: ( $F(1,23)=0.16, p=0.29$ ) independently or overall during the experiment (Group:  $F(1, 22) = 1.91, p=0.18$ , Month:  $F(1, 22) = 17.32, p<0.001$ , Group x Month:  $F(1, 22) = 0.01, p=0.93$ ).



A.



B.



**Figure 3.14 Y-maze data of rats fed control (Con) or western diet (WD) for 12 weeks;**

A. No significant difference was observed between the control and western diet groups in the time the rats spent in the novel arm, each month independently or overall during the experiment.  $n=12$ . Values are mean  $\pm$  SE. B. No significant difference was observed between the control and western diet groups in the number of entries into the novel arm, each month independently or overall during the experiment.  $n=12$ . Values are expressed as mean  $\pm$  SE.

### **3.4. Discussion**

The aims of the present study were to (i) find the time point at which a western diet affects the memory paradigms in rats (ii) investigate the effects of a western diet on neuronal activation and (iii) investigate the effect of western diet consumption on astrocyte number.

After twelve weeks of feeding, male Long Evans rats that consumed a western diet did not gain more weight than the rats that consumed a control diet however, they had significant higher percentage of caloric intake (Figure 3.3) and epididymal fat pad (Figure 3.4). This is consistent with the previous studies in our laboratory showing a significant increase in epididymal fat pad and fasting blood glucose level without changing the body weight, in rats that consumed a 36% fat diet for twelve weeks compared to normal diet (Chen et al., 2010). Given that we observed a significant increase in the body weight of rats in the previous study that consumed the same western diet (SF00-219) for twelve weeks, this difference observed in the result can be due to the differences between the animals within the same strains that respond differently to a high fat diet consumption and the resistance that some animals show within the same strain to induce obesity as identified in the other animal studies (Schemmel et al., 1970, Rothwell et al., 1982, Levin et al., 1989, Levin and Keesey, 1998). Interestingly, S.Akieda-Asai and colleagues in 2013 performed an investigation in which they assessed the metabolic characteristics of a high fat diet resistant rats by giving male Wistar and Sprague-Dawley rats a control and a high fat food (Akieda-Asai et al., 2013). The diet resistant rats although lighter, were not different with diet-induced obese rats in terms of calorie intake, O<sub>2</sub> consumption or locomotor activity. Based on these findings they suggested that the diet resistant rats may be using a specific mechanism that promotes catabolism of excessive fat (Akieda-Asai et al.,

2013). They suggested that suppression of lipogenesis and acceleration of fatty acid  $\beta$ -oxidation in the visceral fat may contribute to the characteristics observed in rats that consumed a western diet but did not show the obesity characteristics (Akieda-Asai et al., 2013). Different response to a higher percentage of fat in diet has been moreover observed in humans studies (Levine et al., 1999). It should be noted that the control diet used in this experiment (SF04-057) was different with the first experiment (AIN-93G). However, the difference observed between the two studies does not seem to be due to the difference in the control diet as although the rats that consumed (AIN-93G) control diet gained an average of  $143.91 \pm 1.51\%$  of their initial body weight at week twelve and rats that consumed (SF04-057) control diet in the current study gained an average of  $138.40 \pm 1.53\%$  of their initial body weight, however, the percentage of initial body weight of rats after consuming a western diet for twelve weeks was  $154.45 \pm 1.65\%$  in the first study compared to  $139.77 \pm 1.70\%$  in the current one.

The present study investigated the effects of western diet on memory paradigms using Y-maze and novel object recognition task in rats. Results showed no significant difference between the control and the western diet group in the time rats spent in the novel arm each month, nor in the number of entries into the novel arm (Figure 3.14). Similarly, the time rats spent exploring the novel object each month in the novel object recognition test and the discrimination ratio were not statistically different between the two groups (Figure 3.13). It is important to note that both paradigms of novel object recognition and Y-maze were carried out every month for three months while the rats were consuming a western and a control diet. This difference observed in the current result and the outcome observed in the previous study (Kosari et al., 2012) in the performance of rats in Y-maze following consumption of a western diet, could be due to multiple reasons. In the present study, rats were first tested in the Y-maze paradigm

after only a month of western diet consumption and no impairment in their performance was observed. It can be argued that longer duration of western diet consumption was needed to develop any impairment. As the task was repeated in the second and third month, it is possible that they may have familiarised themselves with the testing environment and that could potentially affect the result in the following two months. It should be noted that other studies that repeated the Y-maze task after few weeks, observed an impairment in the result (Melnikova et al., 2013) used mazes with different colours, materials and spatial cues. Moreover, each time that the task was repeated, the mazes were placed in different rooms and cleaned by different solutions. However, I used the same maze and the same room to conduct all the experiments. Finally, the differences that exist between the animal body weights can also play a role in this observation.

Investigating the effects of western diet on basal neuronal activity in the present study, revealed that consumption of a western diet for twelve weeks significantly decreased basal c-fos immunoreactivity in the CA2&3 regions of the hippocampus, prelimbic and infralimbic regions, indicating a decreased neuronal activity in those areas. Prelimbic region of prefrontal cortex plays an important role in working memory specifically for visual object and spatial location information. Moreover, prefrontal cortex is connected with the hippocampus, which has long been associated with spatial memory and navigation. Studies showed that a decreased c-fos expression in the prefrontal cortex and the hippocampus may cause short term memory impairment in rats in different spatial memory tasks (Cholvin et al., 2014). A recent study in 2014 showed a significant decrease in c-fos protein expression in the anterior cingulate of rats that consumed a high fat diet for fifteen weeks, however, this study did not investigate other areas of the prefrontal cortex (Chen et al., 2014). It is interesting to note that another

study in 2015 demonstrated that acute consumption of high fat diet for one day activated the neurons in the medial prefrontal cortex (Del Rio et al., 2015) suggesting that acute consumption of high fat diet can act as a reward and reinforcer in that area of the brain (Del Rio et al., 2015).

I have observed a significantly decreased basal c-fos immunoreactivity following consumption of a western diet in the paraventricular nucleus of the thalamus region which has extensive connections with the prefrontal cortex and the hippocampus. Moreover, in the present study, no significant changes were observed in CA1, dentate gyrus, nucleus accumbens and cingulate cortices and regions of the hypothalamus after consumption of a western diet in rats. These results demonstrate that consumption of a western diet for twelve weeks affects neuronal activation in only specific brain regions involved in memory regulation.

Very recently, astrocyte number has been measured in the hypothalamus following a high fat diet and it has been shown that the number of astrocyte in this area has an important homeostatic role in the acute physiologic regulation of food intake (Buckman et al., 2015), however, no study to my knowledge investigated the potential changes that may occur in the number of astrocytes in the regions of the brain important for cognition and memory following a western diet consumption in adult rats. The number of GFAP-positive cells (as a measure of astrocyte number) was decreased significantly in CA1 and dentate gyrus of hippocampus suggesting a decrease in the number of GFAP-positive astrocytes as the result of consumption of a western diet. It is interesting to note that a study in 2015 found that perinatal high fat feeding was linked with changes in astrocyte morphology in CA1 of the hippocampus in rats (Lepinay et al., 2015) and it is important to note that the GFAP marker used is a

cytoskeletal protein and the shape of the astrocytes is linked to cytoskeletal protein expression. No change was observed in the number of astrocytes after three months of western diet consumption in the prefrontal cortex.

The findings show that consumption of a western diet in the settings of this experiment did not affect the memory paradigms within the three months period. Western diet reduced basal neuronal activity in CA2&3 of the hippocampus, infralimbic and prelimbic regions of prefrontal cortex and in the paraventricular nucleus of thalamus but not in paraventricular nucleus and arcuate nucleus of the hypothalamus, nucleus accumbens or CA1 or dentate gyrus of hippocampus or cingulate area of the prefrontal cortex. The finding demonstrate that western diet consumption reduced the number of astrocytes in CA1 and dentate gyrus of the hippocampus but not in CA2&3 or the prefrontal cortex of the brain. Overall, findings suggest that consumption of western diet can influence basal neuronal activity and astrocyte number in the regions of the brains that are involved in memory.

## **4. Chapter 4: Effect of western diet on memory and brain insulin level**

### **4.1. Introduction**

I have shown in chapter three, that consumption of a western diet for three months can change basal neuronal activity in specific brain areas important for memory regulation (see chapter 3 for details). Other studies reported that a change in c-fos expression is dependent on the changes in the level of insulin like growth factor 1 (IGF-1). For example, it has been shown that by blocking the uptake of IGF-1 by brain cells, the exercise-induced increase in c-fos expression was also blocked (Carro et al., 2000). Furthermore, reports have shown that exercise or intracarotid injection of IGF-1 can cause neuronal accumulation of IGF-1 and an identical widespread increase in neuronal c-fos (Carro et al., 2000). It is still unknown if the change in the level of basal c-fos can be partly associated with the possible changes in the level of IGF-1 in the brain in obesity. Changes in the level of brain IGF-1 is important not only due to its correlation with neuronal activity but also studies investigating the role of IGF-1 in memory suggest an important function of this peptide in modulating learning and memory (Trejo et al., 2008, Narimatsu et al., 2009, Zhao et al., 2010). Few studies investigated the changes in the level of IGF-1 in obesity and have reported conflicting results (Relling et al., 2006), and those are mainly focused on the changes that occur in hippocampus (Casadesus et al., 2004). It is unknown if the level of this hormone in specific areas of the brain such as the prefrontal cortex or retrosplenial cortex changes with consumption of higher percentage of fat in diet (The role of IGF-1 in the central nervous system has been discussed extensively in chapter one).

I addressed this question by conducting an experiment to investigate the effect of a western diet on the level of IGF-1 receptor in areas of the brain that are important for memory regulation.

Although hippocampus is an important area in memory regulation, recent evidences support the involvement of other brain areas in memory function. The retrosplenial cortex is located in an important position in the flow of information between the hippocampal formation and the neocortex as explained extensively in chapter 1. The retrosplenial cortex serves as an input structure to the hippocampus and contributes to processing the information pertained to navigation and context. It also, acts as a target for hippocampal output during systems consolidation (Miller et al., 2014). This along with other clinical evidences that show dysfunction of the retrosplenial cortex can lead to early stages of Alzheimer's disease and amnesia, further illustrate the need to study these areas of the brain (Czajkowski et al., 2014, Knight and Hayman, 2014).

Studies showed that prelimbic and infralimbic cortices are important in working memory for visual object and spatial location information (Brito and Brito, 1990, Granon et al., 1994, Seamans et al., 1995, Delatour and Gisquet-Verrier, 1996, Fritts et al., 1998, Ragozzino et al., 1998, Horst and Laubach, 2009). Moreover, I tested the performance of rats in T-maze after consuming a western diet for three months. This can provide the opportunity to further investigate the effect of diet on performance in alternation task in a paradigm that involves food as a reward.



#### **4.1.1. T-maze**

It has been known for a long time that rats will avoid the arm of the T-maze that they have visited before spontaneously (Dember and Fowler, 1958, Dudchenko, 2001) initiated with the observations made by Tolman (Tolman, 1925) and Dennis (Dennis, 1935). This assisted the investigators to examine the rodents exploration behaviour, perception and memory (Dember and Earl, 1957). The reinforced T-maze is also used widely to study the spatial abilities of rodents as it has been shown that reward increases the tendency of the animals to alternate (Zeaman and House, 1951, Walker, 1956, Dember and Fowler, 1958, Dudchenko, 2001, Lalonde, 2002). In reinforced alternation paradigm, first the rodent runs up the stem of the T-maze while one of the cross arms is blocked off. Then the rodent is returned to the start arm and allowed to choose any of the other two arms. When animal enters the arm that has not been entered before, it receives a reward (food pellets). Therefore reinforcement is available only when the rat alternates in the second run. As the rewarded arm differs each time during the choice run, it is not enough for the animal to learn and remember the association between reward and single location (working memory). The rewarded alternation in T-maze is sensitive to dysfunction in hippocampus and other areas of the brain therefore it is a suitable tool to assess the cognitive ability in rats and mice (Deacon and Rawlins, 2006).

## **4.2. Materials and methods**

### **4.2.1. Animals and chow**

20 Hooded-Wistar rats (Monash University, Australia) were housed in groups of two under a light/dark cycle (12 hour/12 hour), in a temperature controlled room (22°C)

with food and water *ad libitum*. Animals were randomly divided into two groups (n=10) and assigned to either a control (Con; SF04-057, (Control for SF00-219) rodent diet, 6% Fat Semi-Pure Rodent Diet including 0.43% total saturated fatty acids; Specialty Feeds, Perth, Australia) or western diet (WD; SF00-219, 21% total fat including 1.80% total saturated fats and 0.15% cholesterol; Specialty Feeds). Animals received the diets for 12 weeks and behavioural testing was conducted at the end of the feeding period. Food intake and body weight were measured twice-weekly. From this, caloric intake was calculated. All experimental protocols were performed in accordance with the prevention of Cruelty to Animals Act 1986 (Australia), the National Health and Medical Research Council (NHMRC) code EA28 and approved by the RMIT University Animals Ethics Committee.

#### **4.2.2. Behavioural testing: T-maze**

##### **4.2.2.1. Apparatus**

Study conducted in a quiet and dimly lit and temperature controlled (21°C) room. T-maze consisted of two runways made of glass positioned in the shape of a T sign. The long part was 20 x 70 x 20 (width/length/height) cm and at each far end of this arm, there was a food dish 4 cm in diameter and 0.5 cm deep. The short arm that had the starting area was 20 x 40 x 20 (width/length/height) cm. Sliding doors allowed the blocking of the arms that could be opened and closed manually. The food dish was positioned in a way that the rat could detect the food in it only in the immediate vicinity of the food. Few pellets of food were used as motivator for the food-restricted rats to run in the maze.

#### **4.2.2.2. Habituation**

All rats were fed sufficient amount of diet to maintain about 85% of their free-feeding body weight and given several days of habituation to the maze to make sure they would run down the stem of the maze to find food pellets. Each habituation took three minutes. In the first habituation, while all the arms of the T-maze were open, food pellets were placed all over the maze to encourage the rat to rundown the maze. The arm in which the rat was entered first (left or right) was monitored and recorded to make sure there is no obvious tendency towards left or right turn in the maze. Next day rats were placed in the maze for 3 minutes again as yesterday and the third habituation on the same day involved putting many food pellets only in the small container at the far end of the two arms (acquisition and sample arm). This procedure was repeated again this time with less food as the reward at the end of the two arms. On day 3 of habituation, the start barrier was introduced. Rat was placed in the maze with start barrier in place and then the barrier was removed and rat was allowed to run in the maze to eat the food pellets at the end of the two arms.

#### **4.2.2.3. Test procedure**

At the start of each trial, few food pellets were placed in each food well and access to one of the arms was blocked (acquisition arm). On the sample run, the rats that have been on food restriction could only enter the open arm (sample arm) to eat the food. They were then picked up and confined to the start area and the blocked arm was opened. In this phase, the rat was allowed to choose one of the arms (sample or acquisition arm). If the animal had alternated and entered the acquisition arm, it could eat the food reward. This procedure has been repeated ten times for each rat (20 runs

including sample and choice run) each day for three days. Sample arm and acquisition arm were changed randomly during the study to avoid any possible bias.

#### **4.2.3. Brain collection**

Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused with freshly prepared ice cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS). After perfusion brains were removed and placed in 4% PFA for 3 hours at room temperature then transferred to 30% sucrose solution. Coronal sections (30µm) thickness were cut on a cryostat (Leica CM 1900 Microsystems Nussloch GmbH, Germany) and stored in cryoprotectant (37.5% sucrose, 1.25% polyvinyl pyrrolidine, 37.5% ethylene glycol in 0.1M PBS) in 24 well plates until required for analysis. Serial coronal sections (30 µm thick) of the brain were cut using a cryostat (Leica CM 1900 Microsystems Nussloch GmbH, Germany). The sections were collected and kept in cryoprotectant (37.5% sucrose, 1.25% polyvinyl pyrrolidine, 37.5% ethylene glycol in 0.1M PBS) in a 24-well plates then processed immunohistochemically to detect IGF-1 receptors using standard immunohistochemical procedures as described below.

#### **4.2.4. Detecting IGF-1 receptors using immunohistochemistry**

To detect IGF-1 receptors, immunohistochemistry was performed using free floating method. Staining commenced by washing the sections in PBS for fifteen minutes (three washes each taking five minutes). Endogenous peroxidase activity was blocked by incubating with 0.5% H<sub>2</sub>O<sub>2</sub> for 30 minutes followed by three washes in PBS for five minutes duration each. Sections then were incubated for 60 minutes in 3% normal goat serum, 0.4% Triton X-100 proceeded by three washes in PBS (five minutes each) and incubation in anti-IGF-1 receptor primary antibody (sc- 712; Santa Cruz Biotechnology, Santa Cruz, California dilution: 1:100) in 2% normal goat serum, 0.2%

Triton X-100 in PBS for 72 hours at 4 °C. Then sections were washed again three times in PBS for five minutes each and were incubated for one hour with biotinylated secondary antibody (1:250, biotinylated goat anti-rabbit antibody) diluted in normal goat serum (2%) followed by another three washes in PBS and a two hours incubation in ABC kits (Vector laboratories, VECTASTAIN, ABC kit) in room temperature. The ABC solution was prepared at least 30 minutes before the incubation. Then sections were washed twice (for ten minutes duration in total) and incubated in 3,3' Diaminobenzidine (DAB) kit (Vector laboratories, DAB Peroxidase kit e Substrate. Cat. No. SK-4100) until the colour of the sections changed and the staining was visible. Before the sections become too dark the reaction was stopped by putting the sections in dH<sub>2</sub>O. Staining for sections of all the three groups were performed under the same condition and had same incubation time in DAB. Sections were mounted on the gelatine coated slides and passed through an increasing gradient of alcohol followed by xylene then they were cover-slipped using Depex mounting medium.

Six images were captured from each region of interest. An IGF-1 receptor analysis was performed using Image J program by measuring the mean grey value of the areas of interest. The averages were calculated and analysis of variance (ANOVA) tests were performed using procedures for general linear models with operations for repeated measures and simple effects.

#### **4.2.5. Statistical analysis**

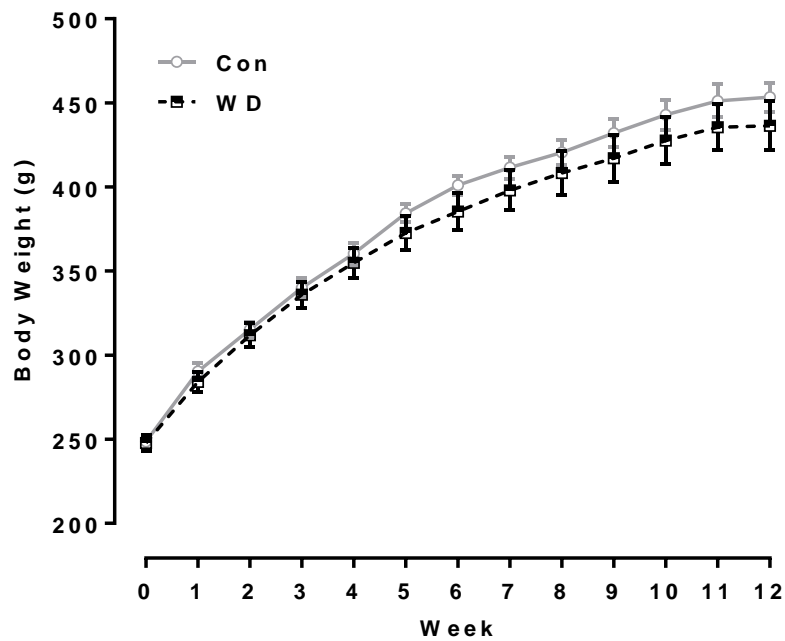
All data were analysed using SPSS for Windows Version 18.0. Body weight and calorie intake were analysed using two-way ANOVA with group and week as factors; when applicable, simple effects for each group were analysed. The epididymal fat pad

weight value was assessed using Student's t-test. IGF-1R density was analysed using two-way ANOVA with group and region as factors; simple effects for each group were determined when applicable.

### **4.3. Results**

#### **4.3.1. Effect of diet on body weight, food and calorie intake and epididymal fat pad**

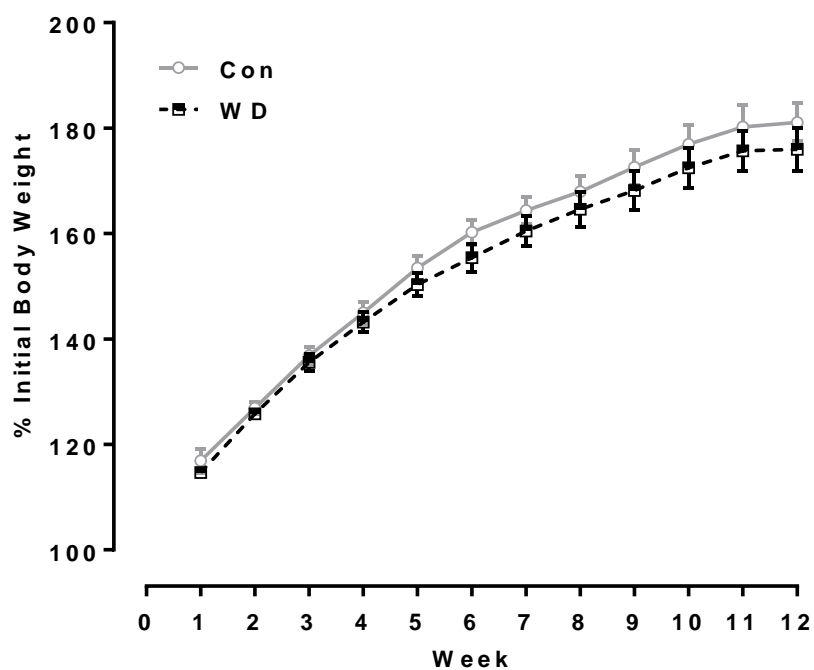
Body weights at the beginning of the dietary feeding regimen were not significantly different among the two groups (Con  $239.35 \pm 2.73$ g, WD  $239.25 \pm 4.88$ g;  $p=0.99$ ). Analysis of the body weight over the 12 experimental weeks demonstrated no significant difference between the two groups (Group:  $F(1,17)=0.94$ ,  $p=0.35$ ; Week:  $F(1,17)=564.70$ ,  $p<0.001$ ; Group x week:  $F(1,17)=0.57$ ;  $p=0.46$ ). Similarly the analysis of the percentage of the initial body weight over the 12 experimental weeks revealed that the only significant differences are between the different weeks in the same group and no significant difference between the two groups was observed (Group:  $F(1,17)=0.96$ ,  $p=0.34$ ; Week:  $F(1,17)=544.78$ ,  $p<0.001$ ; Group x week:  $F(1,17)=0.37$ ;  $p=0.55$ ).



**Figure 4.1** Body weight (g) of rats fed control (Con) or western diet (WD) for 12 weeks;

No significant difference was observed between the two groups in the body weight of rats over the 12 experimental weeks. n=10 (control group was n=9 from week 5).

Values are expressed as mean + SE.



**Figure 4.2 Percentage of the initial body weight of rats fed control (Con) or western diet (WD) for 12 weeks;**

No significant difference was observed between the two groups in the percentage of the initial body weight of rats over the 12 experimental weeks. n=10 (control group was n=9 from week 5). Values are expressed as mean + SE.

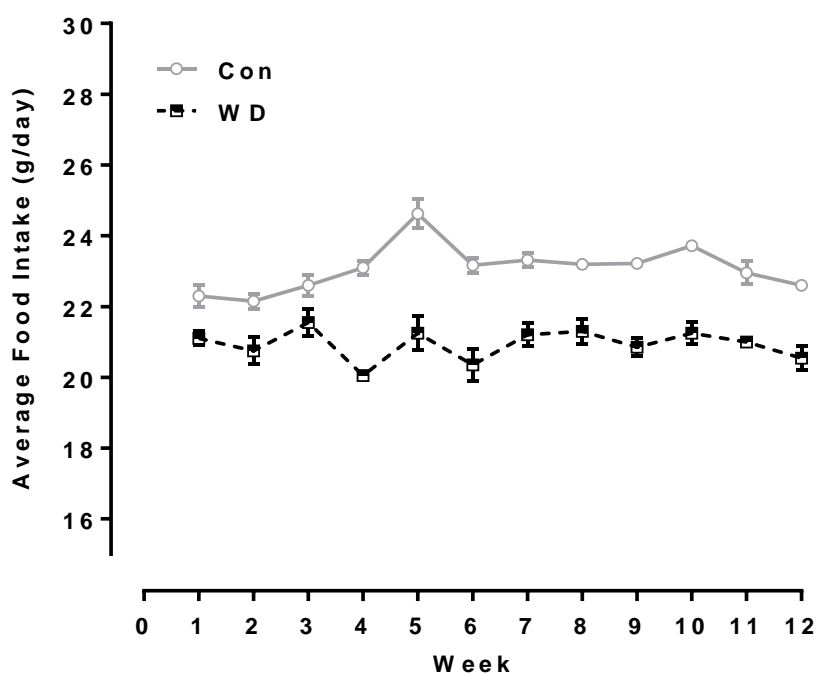


#### **4.3.2. Effect of diet on food and calorie intake and epididymal fat pad**

Average food intake over the entire experiment was significantly different between the groups (Group:  $F(1,17)=42.29$ ,  $p<0.001$ ; Week:  $F(1,17)=4.36$ ,  $p=0.052$ ; Group x week:  $F(1,17)=6.10$ ,  $p=0.02$ ). Average weekly food intake/rat was Con  $23.07\pm0.20$ g and WD  $20.93\pm0.13$ g. Post hoc analysis showed a significant difference between the groups every week (week 1 to 12).

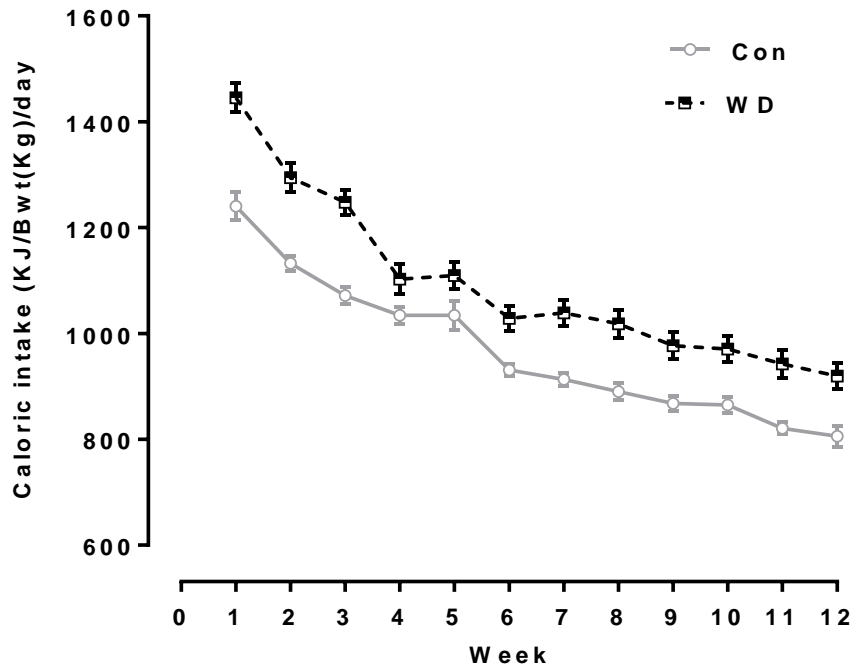
Caloric intake of the two groups was significantly different (Group:  $F(1,17)=21.42$ ,  $p<0.001$ ; Week:  $F(1,17)=667.49$ ,  $p<0.001$ ; Group x week:  $F(1,17)=4.25$ ,  $p=0.055$ ). Further analysis demonstrated a significant exacerbation in calorie intake by rats that received a western diet compared with the control in every week except week 4 and 5.

An analysis of epididymal fat pad revealed no significant increase in the percentage of Fat weight/Body weight of rats that consumed a western diet compared to the control group  $p=0.33$ .



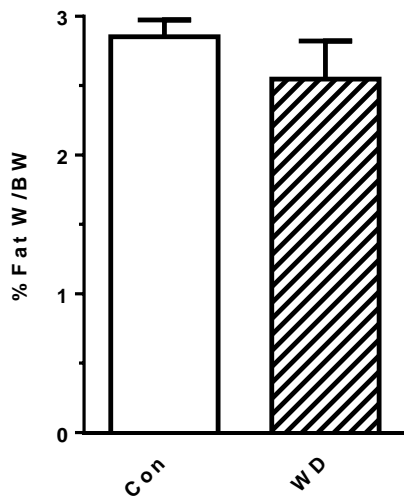
**Figure 4.3 Average food intake (g/day) per rat fed control (Con) or western diet (WD) for 12 weeks;**

Average food intake of the control diet was significantly higher compared to western diet group over the entire experiment (Group:  $F(1,17)=42.29$ ,  $p<0.001$ ; Week:  $F(1,17)=4.36$ ,  $p=0.052$ ; Group x week:  $F(1,17)=6.10$ ,  $p=0.02$ ) and there was a significant difference between the groups every week (week 1 to 12). Average weekly food intake/rat was Con  $23.07\pm0.20$ g and WD  $20.93\pm0.13$ g.  $n=10$ . Values are expressed as mean  $\pm$  SE.



**Figure 4.4 Caloric intake (KJ/Body weight (Kg)/day) of rats fed control (Con) or western diet (WD) for 12 weeks;**

Caloric intake of the western diet group was significantly higher than the control group (Group:  $F(1,17)=21.42$ ,  $p<0.001$ ; Week:  $F(1,17)=667.49$ ,  $p<0.001$ ; Group x week:  $F(1,17)=4.25$ ,  $p=0.055$ ). This significant exacerbation in calorie intake was observed in rats that received a western diet compared with the control group in every week except week 4 and 5.  $n=10$ . Values are expressed as mean  $\pm$  SE.

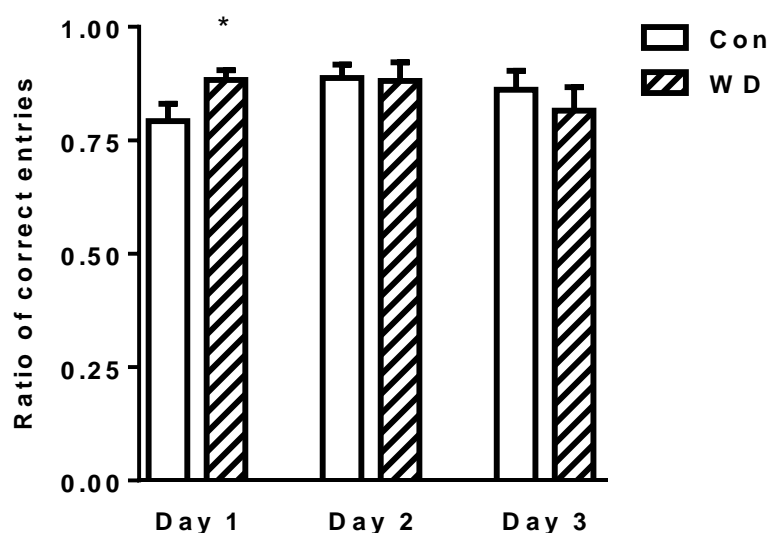


**Figure 4.5 Epididymal fat pad analysis. Percentage of the fat weight/body weight of rats fed control (Con) or western diet (WD) for 12 weeks;**

Analysis of epididymal fat pad revealed no significant increase in the percentage of fat weight/body weight of rats that consumed a western diet compared to the control group  $n=9$ .  $p=0.33$ . Values are expressed as mean  $\pm$  SE.

#### **4.3.3. Effect of diet on T-maze paradigm**

Ratio of correct entries in the T-maze between the two groups was not statistically different. (Group:  $F(1,15)=0.097$ ,  $p=0.76$ , Day:  $F(1,15)=0.00$ ,  $p=0.99$ , Group  $\times$  Day:  $F(1,15)=4.85$ ,  $p<0.05$ ). One-way ANOVA analysis of correct entries of each day separately showed a significantly higher ratio of correct entry by the group consumed a western diet compared to the control group in day 1 ( $F(1,16)=4.66$ ,  $p=0.048$ ) but not in day 2 ( $F(1,16)=0.015$ ,  $p=0.903$ ) or day 3 ( $F(1,16)=0.46$ ,  $p=0.51$ ) of the T-maze paradigm experiment.



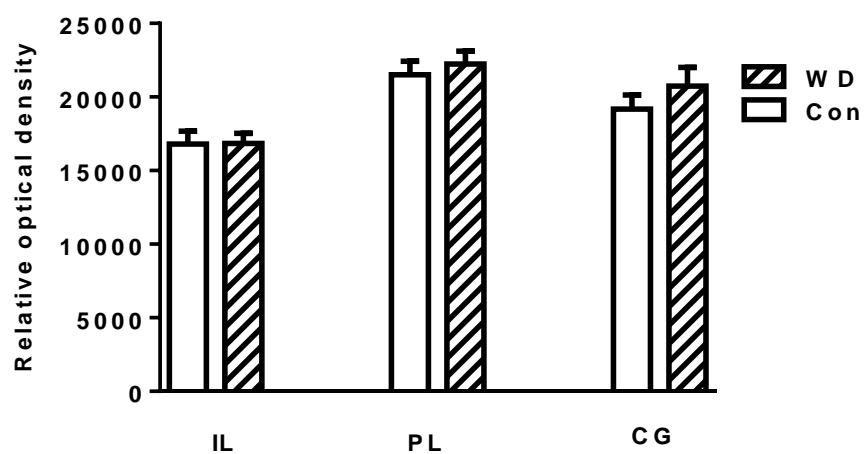
**Figure 4.6 T-maze data of rats fed control (Con) or western diet (WD) diet for 3 months;**

Ratio of correct entries in the T-maze between the two groups was not statistically different. Analysis of correct entries of each day separately showed a significantly higher ratio of correct entry by the group consumed a western diet compared to the control group in day 1 ( $F(1,16)=4.66$ ,  $p=0.048$ ) but not in day 2 ( $F(1,16)=0.015$ ,  $p=0.903$ ) or day 3 ( $F(1,16)=0.46$ ,  $p=0.51$ ) of the T-maze paradigm experiment.  $n=10$ . Values are expressed as mean  $\pm$  SE.

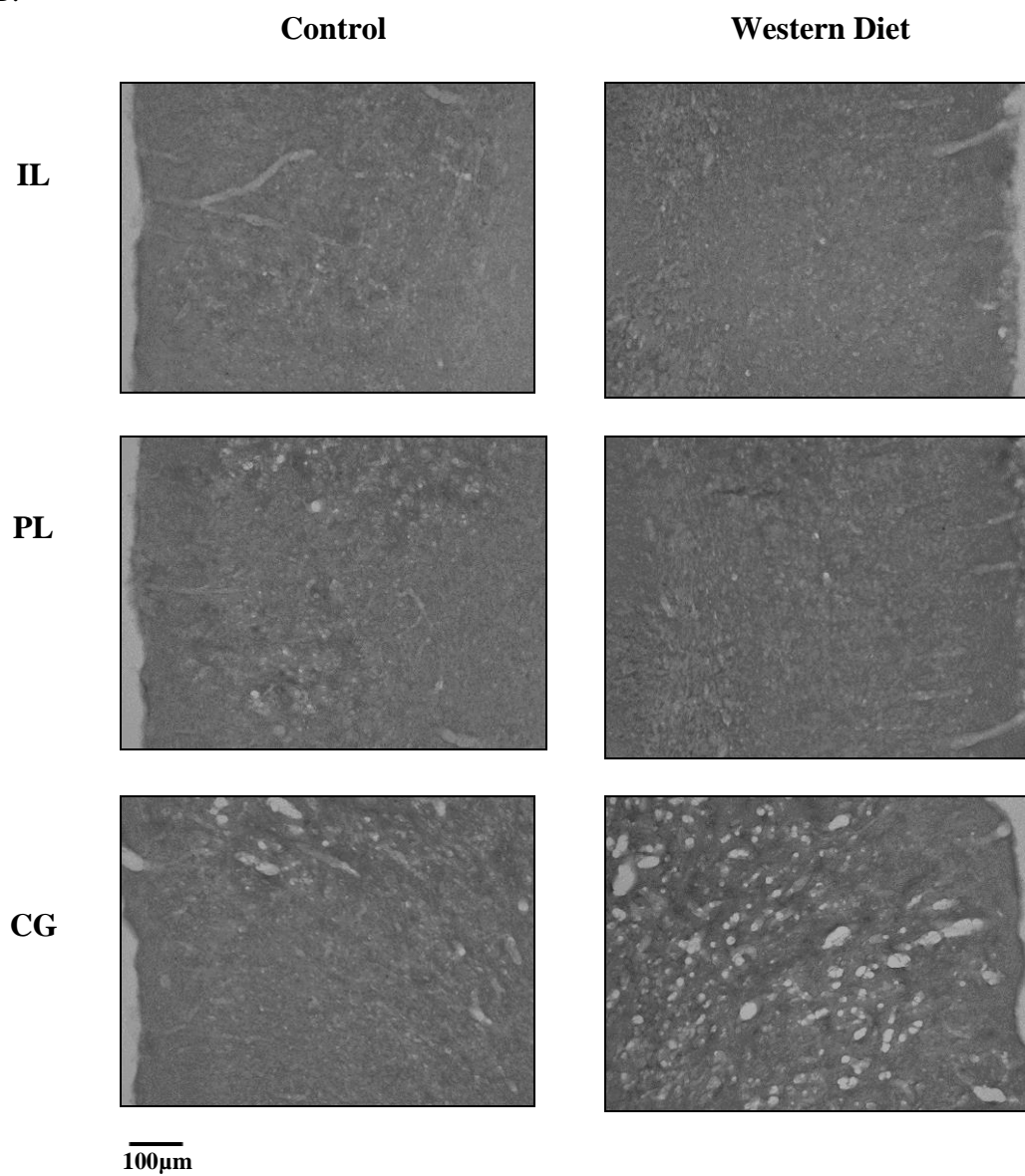
#### **4.3.3.1. Effect of diet on IGF-1R density in the prefrontal cortex**

No significant difference was observed in the number of IGF-1 receptors in the prefrontal cortex of both groups of control and western diet (Group:  $F(1,16)=0.47$ ,  $p=0.50$ , Area:  $F(1,16)=19.75$ ,  $p<0.001$ , Group  $\times$  Area:  $F(1,16)=1.16$ ,  $p=0.30$ ). Analysis of one-way ANOVA revealed no difference between the two groups in each area independently (IL: ( $F(1,17)= 0.002$ ,  $p=0.97$ ), PL: ( $F(1,17)= 0.34$ ,  $p=0.57$ ), CG: ( $F(1,17)= 0.96$ ,  $p=0.34$ )).

A.



B.



**Figure 4.7 A. Relative optical density of IGF-1 receptor staining in infralimbic, prelimbic and anterior cingulate of the prefrontal cortex of rats fed control (Con) or western diet (WD) for 13 weeks. B. Immunohistochemical staining for IGF-1 receptor in the prefrontal cortex subregions;**

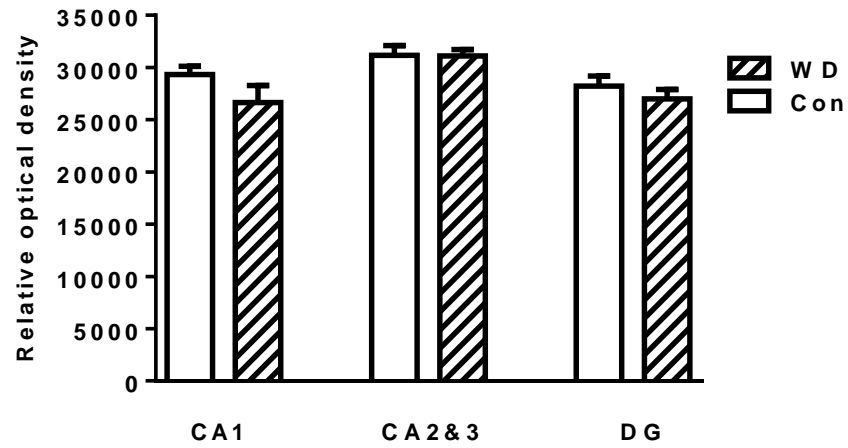
No significant difference was observed in the number of IGF-1 receptors in the prefrontal cortex of the two groups of control and western diet. Similarly no difference was observed between the two groups in each area independently.  $n=10$ .

Values are expressed as mean  $\pm$  SE.

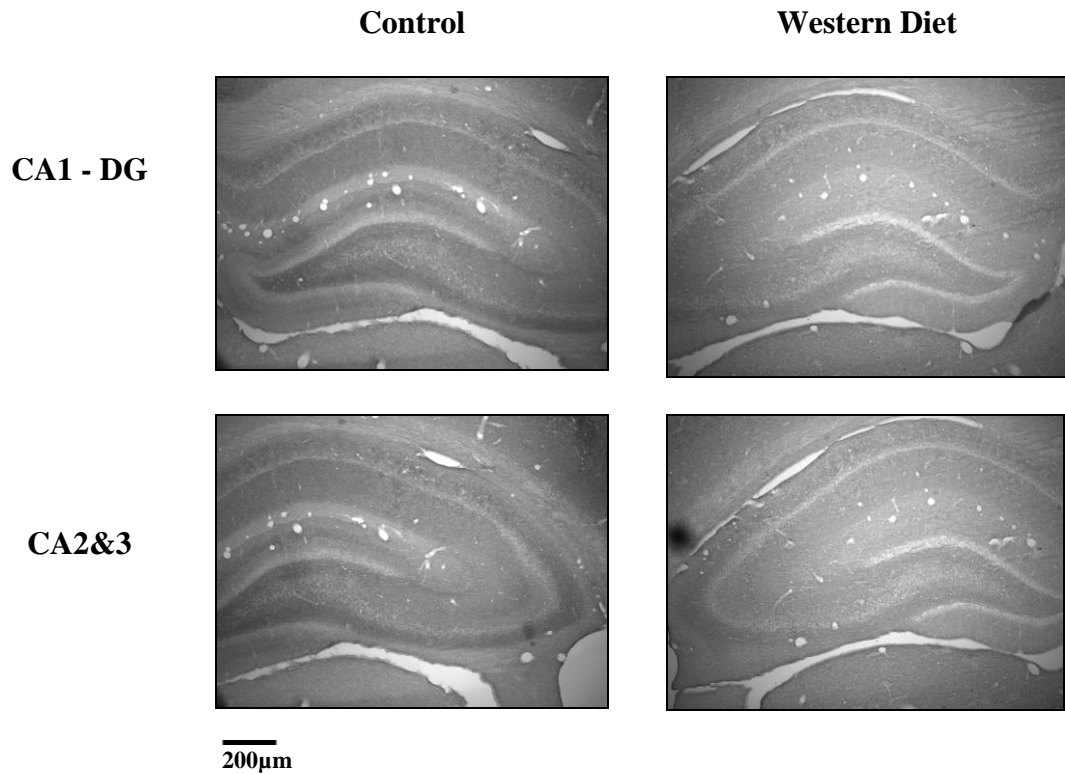
#### **4.3.3.2. Effect of diet on IGF-1R density in the hippocampus**

No significant difference was observed between the number of IGF-1 receptors in the hippocampus of both control and western diet groups (Group:  $F(1,15)=0.007$ ,  $p=0.935$ , Area:  $F(1,15)=0.509$ ,  $p=0.487$ , Group x Area:  $F(1,15)=0.293$ ,  $p=0.597$ ). Analysis of one-way ANOVA revealed no difference between the two groups in each area independently (CA1: ( $F(1,17)=0.023$ ,  $p=0.88$ ), CA2&3: ( $F(1,17)=0.004$ ,  $p=0.951$ ), DG: ( $F(1,16)=0.852$ ,  $p=0.371$ )).

A.



B.



**Figure 4.8 A. Relative optical density of IGF-1 receptor staining in CA1, CA2&3 and dentate gyrus (DG) regions of the hippocampus of rats fed control (Con) or western diet (WD) for 13 weeks. B. Immunohistochemical staining for IGF-1 receptor in the hippocampus subregions;**

No significant difference was observed between the number of IGF-1 receptors in the hippocampus of both control and western diet groups. Similarly no difference

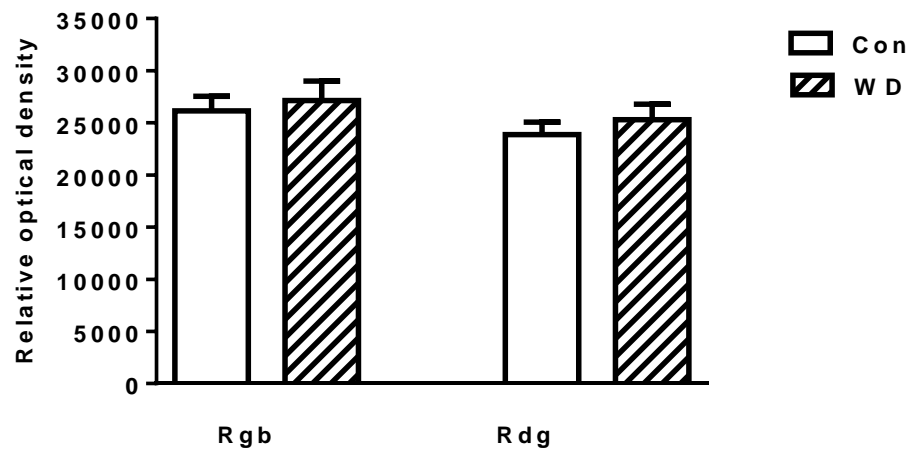


was observed between the two groups in each area independently.  $n=10$ . Values are expressed as mean  $\pm$  SE.

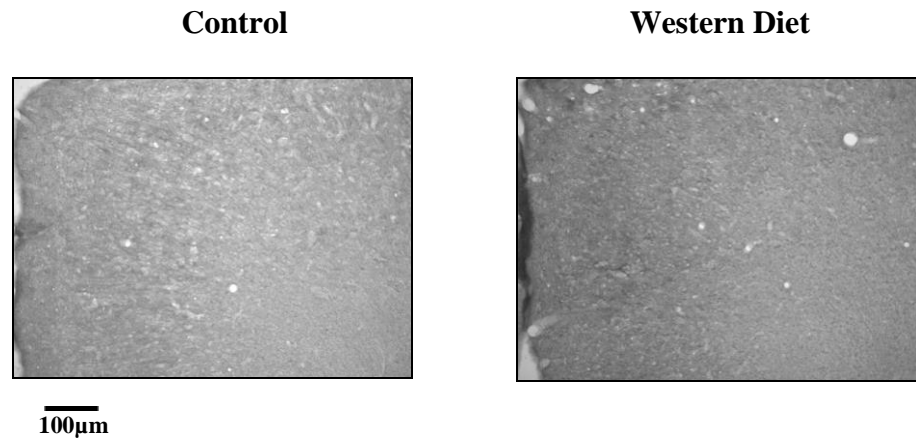
#### **4.3.3.3. Effect of diet on IGF-1R density in the retrosplenial cortex**

No significant difference was observed between the number of IGF-1 receptors in the retrosplenial cortex of both control and western diet groups (Group:  $F(1,12)=0.49$ ,  $p=0.497$ , Area:  $F(1,12)=24.24$ ,  $p<0.001$ , Group  $\times$  Area:  $F(1,12)=2.56$ ,  $p=0.136$ ). Analysis of one-way ANOVA revealed no difference between the two groups in each area independently (Rgb: ( $F(1,13)=0.18$ ,  $p=0.675$ ), Rdg: ( $F(1,15)=0.561$ ,  $p=0.47$ )).

A.



B.



**Figure 4.9 A. Relative optical density of IGF-1 receptor staining in dysgranular cortex (Rdg) and granular b cortex (Rgb) regions of the retrosplenial cortex of rats fed control (Con) or western diet (WD) for 13 weeks. B. Immunohistochemical staining for IGF-1 receptor in the retrosplenial cortex;**

No significant difference was observed between the number of IGF-1 receptors in the retrosplenial cortex of both control and western diet groups. Similarly no difference was observed between the two groups in each area independently. n=6. Values are expressed as mean  $\pm$  SE.

#### **4.4. Discussion**

In this study I have investigated (i) the effects of western diet on T-maze paradigm in adult male Hooded-Wistar rats fed control and western diet and (ii) the changes that occurred in IGF-1 receptor level in the brain as the result of consumption of control and western diets. Consumption of a western diet for thirteen weeks caused a significant increase in caloric intake but no statistically significant increase observed in the body weight of the animals compared with the control group.

In the present study, I have not observed a significant difference in the performance of Hooded-Wistar rats in T-maze paradigm between the group that consumed a western diet and a control diet. Spatial memory impairments have been previously reported in other studies using multi-trial tasks such as water maze and radial arm maze following consumption of a high fat diet (Molteni et al., 2002, Ullrich et al., 2010). Rats that consumed a high fat diet (60% fat) also performed poorly in a modified stone T-maze in which the animals need to learn thirteen correct sequence of left and right in order to reach the goal box and escape the maze (Pistell et al., 2010) but rats that consumed a western diet (41%fat) did not performed poor in the same task (Pistell et al., 2010). There are only two studies conducted recently in 2014 that examined the effect of higher percentage of fat (60% and 45% kcal from fat ) in the diet for various duration on spontaneous alternation task using free-trial T-maze in rodents (Arnold et al., 2014, Peng et al., 2014). Mice that consumed high fat diets in both studies showed an impairment in this memory paradigm (Arnold et al., 2014, Peng et al., 2014). This is consistent with the observation I made previously (discussed in chapter 2) that revealed memory impairment using Y-maze paradigm following consumption of a western diet for twelve weeks by Long Evan rats (Kosari et al., 2012). It is important to note that while Y-maze and the above T-maze paradigms are spontaneous alternation tasks,

forced-trial T-maze task is used in this study. This task is a learned reinforced T-maze that used food pellets as the reward, while the animals were food restricted. The reinforced T-maze version differs with spontaneous alternation (non-reinforced) as in the former the sample run is specified and the alternation is rewarded. Since the task will be repeated successively, the animal learns the correct entry over time. Therefore it can test the working memory of the rodents (Aultman and Moghaddam, 2001). Prefrontal cortex has an important role in this memory as discussed before. To my knowledge except the test I conducted, no study so far examined the effect of a high fat diet or western diet on this task.

In order to investigate further the changes that occur in brain following consumption of a western diet, brain IGF-1 receptor levels were measured using immunohistochemistry. I found no changes in the level of IGF-1 receptor in the hippocampus, the prefrontal cortex and the retrosplenial cortex measured by immunohistochemistry. Several studies reported an important function of IGF-1 in learning and memory. Lupien et al provided evidences on prevention of cognitive impairment in diabetic rats by systemic IGF-1 administration (Lupien et al., 2003) and administration of exogenous IGF-1 has been reported to reverse the impaired spatial learning in mutant mice with low levels of serum IGF-1 (Trejo et al., 2008). Analysis in the prefrontal cortex, hippocampus and retrosplenial cortex of Hooded-Wistar rats that consumed a western diet for thirteen weeks did not show any statistically significant difference in the level of IGF-1 receptor compared to the control group. The fact that there was no change in the expression of IGF-1 receptor does not completely eliminate the chance of reduction in the level of IGF-1 in those areas. Although to my knowledge, no study so far investigated the effect of western diet on IGF-1 receptors in the prefrontal cortex, hippocampus and retrosplenial cortex. Previous studies

investigated the changes that occur in the level of IGF-1 receptor and IGF-1 in other areas of the brain such as choroid plexus following consumption of a high fat diet (Dietrich et al., 2007). One study in 2007 revealed that cafeteria diet can cause a reduction in the passage of systematic IGF-1 across the choroid plexus while reporting no change in the receptor levels (Dietrich et al., 2007). They suggested that, this process may be due to an increase in the level of circulating triglycerides that disrupt the interaction of IGF-1 with the protein that transport IGF-1 (megalin) in choroid plexus (Dietrich et al., 2007). It is still unknown if there is a specific brain area that the western diet targets to influence its effect. Therefore there is a need to investigate the potential regions involve in memory and cognition.

Consistent with these findings, our laboratory previously had shown that 36% fat diet for twelve weeks in male Hooded-Wistar rats caused a significant increase in epididymal fat pad and increase in fasting blood glucose level without changing the body weight, compared with the normal diet (Chen et al., 2010). In this experiment no increase was observed in epididymal fat pad of rats fed a western diet compared with the control group. Other studies using Hooded-Wistar rats showed an increase in the level of epididymal fat pad in the group that consumed 36% fat diet. In the current study, however, I used 21% fat diet which is a characteristic of a western diet used commonly in the current literature. Moreover, it has been shown that some animals with different strains or some within the same strain show resistance to high fat-induced obesity (Levin et al., 1989, Levin and Keesey, 1998). For example when Sprague-Dawley rats fed a high fat diet, only half of the rats developed obesity and the rest resisted (Levin et al., 1987, Levin et al., 1989). Therefore apart from the level of fat and other ingredients in the diet, the differences that exist between the animals are important in their response to change in diet. Therefore, different strains can have

different response to a high fat diet as it seems that Wistar rats may not be prone to obesity as the Long Evan rats are as in this study. 21% fat diet did not cause a significant increase in the epididymal fat pad (Figure 4.5), whereas the same diet used by Long Evan rats significantly increased the epididymal fat pad level as shown in Figure 3.4. In the present study, we had to use Hooded-Wistar rats since Long Evan rats were no longer available in Australia.

Analysis of the mean percentage of the initial body weight at week twelve revealed interesting results. The control group of Long Evan rats had much less mean percentage of initial body weight in the second study compared with the Hooded-Wistar rats while using the same control diet ( $138.40 \pm 1.53\text{g}$  in Long Evan rats versus  $181.11 \pm 3.63\text{g}$  in Hooded-Wistar rats). Mean percentage of initial body weight of western diet group at week 12 in Long Evan rats was  $139.77 \pm 1.70\text{g}$  while in Hooded-Wistar rats, it was  $175.99 \pm 4.03\text{g}$ . Table 4.1 shows some of the ingredients of the food used in the control diet of the first experiment (AIN-93G) and the last two experiments (SF04-057).

The energy intake of rats that consumed a western diet was significantly higher compared to the control group (Figure 4.4) and as discussed the analysis of the epididymal fat revealed no significant difference between the groups, therefore, the question arises that how the excess calorie intake is used if they have not turned into fat and body weight. Animals were checked daily and weighed twice a week and no significant difference observed in their activities and movement. It is interesting to note that S.Akieda-Asai and colleagues in 2013 performed an investigation in which they assessed the metabolic characteristics of a high fat diet resistant rats by giving male Wistar and Sprague-Dawley rats a control and a high fat food (Akieda-Asai et al.,

2013). The diet resistant rats although lighter, were not different with diet-induced obese rats in terms of calorie intake, O<sub>2</sub> consumption or locomotor activity. Based on these findings they suggested that the diet resistant rats may be using a specific mechanism that promotes catabolism of excessive fat (Akieda-Asai et al., 2013). They suggested that suppression of lipogenesis and acceleration of fatty acid  $\beta$ -oxidation in the visceral fat may contribute to the characteristics observed in rats that consumed a western diet but did not show the obesity characteristics (Akieda-Asai et al., 2013), however, further research is needed to fully understand this difference.

Analysis of Diet	<b>AIN-93G</b>	<b>SF04-057</b>	<b>SF00-219</b>
	<b>Control Diet</b>	<b>Control diet (For SF00-219)</b>	<b>Western Diet</b>
Protein	18.7%	19.00%	19.00%
Fat	7.0%	6.00%	21.00%
Total Saturated Fatty Acids	1.05%	0.43%	1.80%
Total n3 Fatty Acid	0.55%	0.90%	0.35%
Total n6 Fatty Acid	Not available	1.30%	0.41%
Fibre	5.0%	-	-
Crude Fibre	-	4.70%	4.70%
Percentage of total calculated digestible energy from lipids	16.4%	21.00%	40.00%
Percentage of total calculated digestible energy from protein	18.8%	14.00%	17.00%

**Table 4.1 Diet composition of control (AIN-93G), control (SF04-057) and western diet (SF00-219).**



## **5. Chapter 5: Conclusion**

This study is the first to report memory impairment using a Y-maze paradigm (which is a one trial-one test procedure) as the result of higher percentage of fat in diet in rats (Kosari et al., 2012). Long Evan rats that consumed western (21% fat) or high fat diet (60% fat) for three months showed an impairment in their spatial memory using Y-maze task. Previously, spatial memory impairments have been reported using multi-trial tasks as the result of change in the diet. However, it is interesting to note that I demonstrated that consumption of a high fat diet (60% fat) impaired spatial memory, independently of changes in body weight or blood pressure. I further investigated the effects of diet on memory function in the same groups of rats using novel object recognition tasks. Results revealed no significant difference in the working memory performance of rats that consumed control, western or high fat diets for three months using novel object recognition task. Furthermore, in order to analyse the performance of rats in T-maze paradigm following the consumption of western and control diets for three months, Hooded-Wistar rats were used. I have not observed a significant difference in the performance between the groups that consumed a western diet (21% fat) and a control diet (6% fat).

In order to study how high fat and western diet consumption can affect memory, I have investigated the changes that occur in areas of the brain important for memory following the consumption of those diets in rats. Results revealed a significant increase in the level of acetylcholinesterase (an enzyme in charge of breakdown of acetylcholine) in the striatum of the rats that consumed a high fat diet (60% fat) compared to rats received a control diet. Further analysis in this study on the effect of high fat and western diet on cholinergic activity revealed no difference in the number

of choline acetyltransferase (ChAT)-positive cells (an enzyme responsible for production of acetylcholine) in the medial septum and vertical limb of the diagonal band of the rats that consumed any of the three diets. This observation together with the fact that no change was also observed in the level of acetylcholinesterase in the hippocampus of rats that consumed western and high fat diet for twelve weeks, suggest that acetylcholine level in hippocampus may not play a critical role in the observed spatial memory impairment.

Investigating the effects of western diet on neuronal activity in another groups of Long Evan rats revealed that consumption of a western diet for twelve weeks significantly decreased basal c-fos immunoreactivity in the CA2&3 regions of the hippocampus, prelimbic and infralimbic regions of the prefrontal cortex. This indicated a decreased neuronal activity in those areas. I have observed a significantly decreased basal c-fos immunoreactivity following consumption of a western diet in the paraventricular nucleus of thalamus which has extensive connections with the prefrontal cortex and hippocampus. These results demonstrate that consumption of a western diet for twelve weeks affects neuronal activation in only specific brain regions involved in memory regulation.

Evidence suggest that there may be a possible link between the neuronal activity and Insulin-like growth factor 1 (IGF-1) in the brain as the same pattern of neuronal accumulation of IGF-1 and an identical widespread increase in neuronal c-fos (a marker of neuronal activity) were observed after exercise or intracarotid injection of IGF-1. Interestingly, by blocking the uptake of IGF-1 by brain cells, the exercise-induced increase in the c-fos expression was also blocked (Carro et al., 2000). Therefore, I investigated the changes in the level of IGF-1 receptor in the hippocampus, prefrontal

cortex and retrosplenial cortex in the group of Hooded-Wistar rats that consumed western or control diet for three months. Result revealed no significant differences between the two groups. However, this result does not completely eliminate the chance of reduction in the level of IGF-1 in those areas and further investigation is needed.

This is the first study that investigated the potential changes that may occur in the number of astrocytes in the regions of the brain important for cognition and memory following western diet consumption in adult rats. The immunohistochemistry results revealed that the number of glial fibrillary acidic protein (GFAP) positive cells (as an astrocyte marker) was decreased significantly in CA1 and dentate gyrus of the hippocampus of rats that consumed a western diet for three months. These results suggest a decrease in the number of astrocytes as the result of consumption of a western diet. However, no change was observed in the GFAP immunohistochemistry staining after three months of western diet consumption in the areas of the prefrontal cortex.

In conclusion, this research demonstrated an impairment of spatial memory in rats following the consumption of a western or high fat diet for three months. Further investigation revealed that consumption of these diets is associated with changes in specific areas of the brain including those that are involved in memory regulation. These changes include the increased level of acetylcholinesterase in the striatum, the reduced number of astrocytes in CA1 and dentate gyrus of the hippocampus besides the decreased number of basal neuronal activity in CA2&3 region of the hippocampus, prelimbic and infralimbic areas of the prefrontal cortex and in the paraventricular nucleus of thalamus in rats following consumption of high fat or western diets. Overall, these results suggest that higher percentage of fat in diet can affect specific regions of

the brain that are important for memory regulation through various mechanisms and consequently may affect the memory. Further studies are necessary to elucidate the precise mechanisms that are involved in the association between the consumption of a western diet and changes in the brain that consequently may affect memory function. Those studies may include investigating the effect of inflammation, examining the effect of insulin resistance and various fatty acid compositions on different brain areas that have important role for memory regulation in control and high fat fed rats.

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